



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/63, A61K 48/00	A1	(11) International Publication Number: WO 98/37207 (43) International Publication Date: 27 August 1998 (27.08.98)
<p>(21) International Application Number: PCT/GB98/00551</p> <p>(22) International Filing Date: 20 February 1998 (20.02.98)</p> <p>(30) Priority Data: 9703633.9 21 February 1997 (21.02.97) GB</p> <p>(71) Applicant (for all designated States except US): IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): HICKSON, Ian, David [GB/GB]; 131 Windmill Road, Oxford OX3 7DN (GB). EDWARDS, Susan, Nicola [GB/GB]; 26 Brassey Road, Winchester, Hants SO22 6SB (GB).</p> <p>(74) Agent: BASSETT, Richard; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
<p>(54) Title: ANTITUMOR GENETIC CONSTRUCTS</p> <p>(57) Abstract</p> <p>A genetic construct comprising a promoter element responsive to a tumour suppressor gene product and a cytotoxic gene wherein the promoter element is substantially inactive in a cell which contains said tumour suppressor gene product encoded by a wild type tumour suppressor gene and is active in a cell which contains a variant said tumour suppressor gene product encoded by a mutant tumour suppressor gene, or does not contain said tumour suppressor gene product. The genetic construct is useful in treating cancer.</p> <div data-bbox="698 1092 1429 1386"><p>0 2 4 6 10 15 ug wtp53 plasmid</p></div>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

ANTITUMOR GENETIC CONSTRUCTS

The present invention relates to cancer therapy, and in particular to the treatment of cancer using gene therapy.

5

Cancer is a major killer. Although much progress has been made in understanding the molecular basis of cancer there remains the need for new and improved methods of cancer treatment.

10 Many cancers are known to be associated with mutations in so-called tumour suppressor genes. Tumour suppressor genes include p53, Rb, FAP, DCC, NF1, NF2, WT1, BRCA1, BRCA2 and DPC4. The tumour suppressor gene products such as p53 and Rb are known to modulate transcription of various genes.

15

Hochhauser *et al* (1992) *J. Biol. Chem.* **267**, 18961-18965 describes the cloning and characterization of the 5' flanking region of the human topoisomerase II α gene.

20 Mercer *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1958-1962 shows that wild-type p53 down-regulates PCNA (proliferating cell nuclear antigen) expression. However, the situation is complex since, for example, Morris *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 895-899 indicates that p53 transcriptionally activates the human PCNA promoter.

25

Santhanam *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7605-7609 describes the differential effect of wild-type and mutant p53 on the repression of various promoters.

30 Ginsberg *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9979-9983 shows

that wild-type p53 can down-modulate the activity of various promoters.

Liu & Berk (1995) *Mol. Cell. Biol.* **15**, 6474-6478 describes work which suggests that p53 acts through interactions with transcription factors TFIIB
5 and TFIID.

Isaacs *et al* (1996) *J. Biol. Chem.* **271**, 16741-16747 describes experiments which analyse the regulation of the human topoisomerase II α gene promoter in confluence arrested cells.

10

Sandri *et al* (1996) *Nucl. Acids Res.* **24**, 4464-4470 describes work which shows that p53 regulates the minimal promoter of the human topoisomerase II α gene.

15 Cano *et al* (1996) *Int. J. Cancer* **65**, 254-262 describes that the expression pattern of the cell adhesion molecules E-cadherin, P-cadherin and $\alpha_6 \beta_4$ integrin is altered in pre-malignant skin tumours of p53-deficient mice.

Linardopoulos *et al* (1995) *Cancer Research* **55**, 5168-5172 describes
20 deletion and altered regulation of *p16^{INK4a}* and *p15^{INK4b}* in undifferentiated mouse skin tumours.

Chin *et al* (1992) *Science* **255**, 459-462 describes repression of the multidrug resistance (MDR1) gene by wild-type, but not mutant, p53.

25

Sandri *et al* (1996) *Brit. J. Cancer* (1996) **73**, 1518-1524 describes the differential expression of the topoisomerase II α and II β genes in human breast cancers.

30 WO 97/12970 describes anti-tumour vector constructs and methods.

An object of the present invention is to provide new and improved cancer therapies.

A first aspect of the invention provides a genetic construct comprising a promoter element responsive to a tumour suppressor gene product and a
5 cytotoxic gene wherein the promoter element is substantially inactive in a cell which contains said tumour suppressor gene product encoded by a wild type tumour suppressor gene and is active in a cell which contains a variant said tumour suppressor gene product encoded by a mutant tumour
10 suppressor gene, or does not contain said tumour suppressor gene product.

By a "genetic element responsive to a tumour suppressor gene product" we include those genetic elements (either DNA or RNA, but usually DNA) which either directly or indirectly respond to a wild-type tumour
15 suppressor gene product to cause suppression of the expression of a gene to which said element is operatively linked.

By "directly respond" we mean that the tumour suppressor gene product and the genetic element interact directly.

20

By "indirectly respond" we mean that the tumour suppressor gene product and the genetic element interact indirectly *via* one or more additional factors.

25 In either case the effect is that, when present in a cell which contains a tumour suppressor gene product encoded by a wild type tumour suppressor gene the genetic construct is substantially unable to express the said cytotoxic gene whereas when present in a cell which contains a variant tumour suppressor gene product encoded by a mutant tumour suppressor
30 gene, or when present in a cell which lacks said tumour suppressor gene

product, the genetic construct is able to express said cytotoxic gene.

It will be appreciated that many mutations in a tumour suppressor gene will lead to the production of a tumour suppressor gene product which is substantially incapable of suppressing the activity of the said promoter element. It is these mutations which lead to the variant tumour suppressor gene products as defined in the first aspect of the invention.

Preferably, when present in a normal cell which contains wild-type copies of the given tumour suppressor gene the genetic construct expresses substantially no cytotoxic gene product, or at least a very low level compared to the level of expression of said cytotoxic gene product in a tumour cell wherein both copies of the tumour suppressor gene are mutated or absent (and therefore inactive).

Most preferably the level of expression of the cytotoxic gene product when the genetic construct is present in non-tumour cells is such that there is substantially no toxicity to said non-tumour cells due to the cytotoxic gene product.

Preferably the level of expression of the cytotoxic gene in the tumour cell is at least three times the level in the normal cell; more preferably the level in tumour cell is at least ten times the level in the normal cell; still more preferably it is at least fifty times, and most preferably it is at least one hundred times.

By "cytotoxic gene" we mean a gene which encodes a molecule (either polypeptide or RNA) having a directly or indirectly cytotoxic function.

By "directly or indirectly" cytotoxic, we mean that the molecule encoded

by the gene may itself be toxic (for example ricin; interferon-gamma (IFN- γ); ribonuclease; deoxyribonuclease; *Pseudomonas* exotoxin A) or it may be metabolised to form a toxic product, or it may act on something else to form a toxic product. The sequence of ricin cDNA is disclosed in
5 Lamb *et al* (1985) *Eur. J. Biochem.* **148**, 265-270 incorporated herein by reference. The sequence of cDNAs which encode IFN- γ and other suitable cytotoxic genes are available in the GenBank/EMBL nucleotide sequence database.

10 In relation to the indirectly cytotoxic gene, the gene may encode an enzyme, the enzyme being one that converts a relatively non-toxic prodrug to a toxic drug. The enzyme cytosine deaminase converts 5-fluorocytosine (5FC) to 5-fluorouracil (5FU) (Mullen *et al* (1922) *PNAS* **89**, 33); the herpes simplex enzyme thymidine kinase sensitises cells to treatment with
15 the antiviral agent ganciclovir (GCV) or aciclovir (Moolten (1986) *Cancer Res.* **46**, 5276; Ezzedine *et al* (1991) *New Biol* **3**, 608). The cytosine deaminase of any organism, for example *E. coli* or *Saccharomyces cerevisiae*, may be used.

20 Thus, in a preferred embodiment of the invention, the gene encodes a cytosine deaminase which is able to convert 5-fluorocytosine (5FC) to 5-fluorouracil.

In another preferred embodiment of the invention, the gene encodes
25 thymidine phosphorylase (TP). In humans TP is identical to platelet-derived endothelial cell growth factor (PD-ECGF). TP enhances cell killing by the prodrug 5'-deoxy-5-fluorouridine (5'-DFUR) as disclosed in Patterson *et al* (1995) *Br. J. Cancer* **72**, 669-675.

30 In a further preferred embodiment the gene encodes thymidine kinase

which is able to active ganciclovir or acyclovir.

Other examples of pro-drug/enzyme combinations include those disclosed by Bagshawe *et al* (WO 88/07378), namely various alkylating agents and the *Pseudomonas* spp. CPG2 enzyme, and those disclosed by Epenetos & Rowlinson-Busza (WO 91/11201), namely cyanogenic pro-drugs (for example amygdalin) and plant-derived β -glucosidases. The nitroreductase/CB1954 system described by Bridgewater *et al* (1995) *Eur. J. Cancer* 31A, 2362-2370 is another example of an enzyme/prodrug combination suitable for use in the invention.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumour cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form (see, for example, D.E.V. Wilman "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions* 14, 375-382 (615th Meeting, Belfast 1986) and V.J. Stella *et al* "Prodrugs: A Chemical Approach to Targeted Drug Delivery" *Directed Drug Delivery* R. Borchardt *et al* (ed.) pages 247-267 (Humana Press 1985)).

The enzymatic activation of prodrugs results in a large number of drug molecules generated per conjugate molecule which can then diffuse to tumour regions in which the tumour cell may not have received the genetic construct. Furthermore, not all of the tumour cells in a given population need to have received the genetic construct in order to be affected by the cytotoxic agent. This phenomenon is known as the "bystander" effect and is well known in the art of targeted enzyme/prodrug therapy.

Enzymes that are useful in this embodiment of the invention include, but

are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as abzymes, can be used to convert the prodrugs of the invention into free active drugs [see, eg R J Massey, *Nature*, 328, pp. 457-458 (1987)]. The genes and cDNAs encoding at least some of these enzyme and abzyme are known in the art.

Similarly, the prodrugs of this invention include, but are not limited to, the above-listed prodrugs, eg phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted by the enzyme of the conjugate into the more active, cytotoxic free drug. Examples of

cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, etoposide, teniposide, adriamycin, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, cis-platinum and cis-platinum analogues, bleomycins, 5 esperamicins [see US Patent No 4,675,187], 5-fluorouracil, melphalan and other related nitrogen mustards.

It will be appreciated, as is discussed more fully below, that patients who are administered genetic constructs (or other means of the invention which 10 express the cytotoxic gene) which contain an indirectly cytotoxic gene encoding an enzyme are also administered a cognate prodrug for activation by the enzyme.

In a further embodiment the gene delivered to the target cell encodes a 15 ribozyme capable of cleaving targeted RNA or DNA. The targeted RNA or DNA to be cleaved may be RNA or DNA which is essential to the function of the cell and cleavage thereof results in cell death.

Ribozymes which may be encoded in the genomes of the viruses or virus- 20 like particles herein disclosed are described in Cech and Herschlag "Site-specific cleavage of single stranded DNA" US 5,180,818; Altman *et al* "Cleavage of targeted RNA by RNase P" US 5,168,053, Cantin *et al* "Ribozyme cleavage of HIV-1 RNA" US 5,149,796; Cech *et al* "RNA ribozyme restriction endoribonucleases and methods", US 5,116,742; 25 Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endonucleases and methods, US 5,093,246; and Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods; cleaves single-stranded RNA at specific site by transesterification", US 4,987,071, all incorporated herein by reference.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

Preferably, the genetic construct is adapted for delivery to a cell, preferably a human cell. More preferably, the genetic construct is adapted for delivery to a cell in an animal body, more preferably a mammalian body; most preferably it is adapted for delivery to a cell in a human body.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* 16, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers are in a quiescent, non-receptive stage of cell growth or, at least, are dividing much less rapidly than the tumour cells. Retroviral DNA constructs which contain a promoter segment which comprise a promoter element responsive to a tumour suppressor gene product and a cytotoxic gene as defined may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a *neo^R* gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μ m pore-size filter and stored at -70°. For the

introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 μ g/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml.

Alternatively, as described in Culver *et al* (1992) *Science* **256**, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells.

Targeted retroviruses are also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into preexisting viral *env* genes (see Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a review of this and other targeted vectors for gene therapy).

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nässander *et al* (1992) *Cancer Res.* **52**, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available (see Table for examples). For the preparation of immuno-liposomes MPB-PE (N-[4-(p-

maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

Table

Target	Binding moiety	Disease
Truncated EGFR	anti-EGFR mAb	Gliomas
Idiotypes	anti-id mAbs	B-cell lymphomas
EGFR (<i>c-erbB1</i>)	EGF, TGF α anti-EGFR mAb	Breast cancer
<i>c-erbB2</i>	mAbs	Breast cancer
IL-2 receptor	IL-2 anti-Tac mAb	Lymphomas and leukaemias
IL-4 receptor	IL-4	Lymphomas and leukaemias

	IL-6 receptor	IL-6	Lymphomas and leukaemias
	MSH (melanocyte-stimulating hormone)	α -MSH	Melanomas
5	Transferrin receptor (TR)	Transferrin anti-TR mAb	Gliomas
	gp95/gp97	mAbs	Melanomas
	p-glycoprotein cells	mAbs	drug-resistant
	cluster-1 antigen (N-CAM)	mAbs	Small cell lung carcinomas
10	cluster-w4	mAbs	Small cell lung carcinomas
	cluster-5A	mAbs	Small cell lung carcinomas
	cluster-6 (LeY)	mAbs	Small cell lung carcinomas
	PLAP (placental alkaline phosphatase)	mAbs	Some seminomas Some ovarian; some non-small cell lung cancer
15	CA-125	mAbs	Lung, ovarian
	ESA (epithelial specific antigen)	mAbs	carcinoma
	CD 19, 22, 37	mAbs	B-cell lymphoma
	250 kDa proteoglycan	mAbs	Melanoma
20	p55	mAbs	Breast cancer
	TCR-IgH fusion	mAbs	Childhood T-cell leukaemia
25	Blood gp A antigen (in B or O individuals)	mAbs	Gastric and colon tumours

It will be appreciated that monoclonal antibodies or other molecules that

bind to tumour cell surface antigens are useful in targeting the DNA construct of the invention, for example as part of an immunoliposome.

Monoclonal antibodies which will bind to many of these antigens are
5 already known but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to
10 selected antigens may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and Applications*", J G R Hurrell (CRC Press, 1982).

15 Chimaeric antibodies are discussed by Neuberger *et al* (1988, 8th International Biotechnology Symposium Part 2, 792-799).

Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into
20 the framework of human antibodies. Such "humanized" antibodies, or fragments thereof, are preferred as they may give rise to a lower anti-antibody reaction than rodent antibodies.

The variable heavy (V_H) and variable light (V_L) domains of the antibody
25 are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody
30 (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and F(ab')₂ fragments are "bivalent". By "bivalent" we mean that the said antibodies and F(ab')₂ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

Other molecules immunologically reactive with the target cell surface molecule are also useful in this aspect of the invention and include, for example minimal recognition units (MRU) and complementarity determining regions.

5

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). In the first of these methods a
0 polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA via electrostatic interactions with the phosphate backbone.
5 The adenovirus, because it contains unaltered fibre and penton proteins, is internalized into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present
0 within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by
5 conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to
0 bind their cognate receptor and to mediate efficient iron transport into the

cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse a tumour with the suitable delivery vehicle comprising the genetic construct for a period of time; additionally or alternatively the delivery vehicle or genetic construct can be injected directly into accessible tumours.

It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* 6, 1129-1144.

Thus, it will be appreciated that a further aspect of the invention provides a composition comprising genetic construct as defined in the invention and means for introducing said genetic construct into a cell, preferably the cell of an animal body.

Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* 2, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* 274, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

It is particularly preferred if the tumour suppressor gene is p53. The p53 gene is known to be mutated or deleted in over 50% of human cancers.

The seven most common human tumour-derived p53 mutants are His175, Gln248, Trp248, Ser249, His273, Trp282 and Cys273. The distribution of p53 mutations identified in various tumour types is described in

Holstein *et al* (1991) *Science* 253, 49-53 and de Fromental & Soussi (1992) *Genes, Chromosomes & Cancer* 4, 1-15.

It is preferred if the human tumour to be treated has a mutant p53 wherein
5 the p53 protein has a mutation in the DNA binding domain (residues 102-292). Missense point mutations in this domain are associated with the development of human cancers. Human cancer cells which have p53 Val138 or p53 His273 mutations are particularly suited to treatment with the genetic constructs of the present invention.

10.

Several promoters are known that are regulated either directly or indirectly by p53. Suitably, in the present invention, the promoters useful in the genetic construct of the first aspect of the invention are those promoters which are substantially repressed by wild-type p53 gene products but are
15 active in the presence of a variant p53 gene product encoded by a mutant p53 gene or in the absence of any p53 gene product. The product of the p53 gene may be absent due to a deletion in both copies of the p53 gene or by mutations in the p53 gene which prevent expression of its product or a combination of deletion and mutation.

20

The promoters described in Santhanam *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88, 7605-7609 and in Ginsberg *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88, 9979-9983 may also be suitable for use. In other words, the promoter may be any one of the promoters from *c-fos*, β -actin, p53,
25 *hsc70*, *c-jun* and IL-6 or a suitable fragment or portion or variant thereof which retains the appropriate activity.

It is also particularly preferred if the promoter element is one which is active in proliferating but is substantially inactive in non-proliferating
30 cells. Preferably, in relation to the genetic construct, the level of

expression of the cytotoxic gene in a proliferating is at least three times the level in a non-proliferating cell; more preferably, the level is at least ten times, still more preferably, it is at least fifty times.

- 5 In this context, a proliferating cell is one which is actively going through the cell cycle, ie is not in G_0 . Suitably, the promoter distinguishes between proliferating cells and quiescent cells. Quiescent cells are out of cycle or are G_0 cells.
- 10 It is particularly preferred if the promoter element of the genetic construct is the human topoisomerase II α gene promoter or a portion thereof.

It is possible that proliferative cell-specific or selective expression can be obtained using promoters which drive expression of cell-cycle genes such
15 as cyclin and PCNA. These may be used in conjunction with the promoter element of the genetic construct of the invention to confer proliferative cell selectivity.

The cloning and characterization of the 5' flanking region of the human
20 topoisomerase II α gene is described in Hochhauser *et al* (1992) *J. Biol. Chem.* **267**, 18961-18965, incorporated herein by reference, and the nucleotide sequences reported in Hochhauser *et al* are present in the Gen BankTM/EMBL Data Bank with accession number X66794. Further studies on the regulation of the human topoisomerase II α gene promoter
25 are described in Isaacs *et al* (1996) *J. Biol. Chem.* **271**, 16741-16747, incorporated herein by reference.

Preferably, the promoter element comprises the polynucleotide described in Figure 8. (This is the same as Figure 1 in Isaacs *et al* (1996) *J. Biol.*
30 *Chem.* **271**, 16741-16747).

Preferably the promoter element comprises the polynucleotide defined by the sequence -500 to +93 in Figure 8; more preferably the promoter element comprises the polynucleotide defined by the sequence -400 to +93 in Figure 8; and still more preferably the promoter element comprises the polynucleotide defined by the sequence -200 to +93 in Figure 8.

It is preferred that the promoter element comprises at least the polynucleotide defined by the sequence -144 to +93 in Figure 8.

The transcription start site in Figure 8 is nucleotide +1. Features which make the topoisomerase II α promoter particularly attractive for use in the invention are (a) its very small size; and (b) that two different regulatory processes act independently on such a short fragment of DNA, that is to say that the topoisomerase II α promoter is active in proliferating cells but not in non-proliferating cells, and the promoter is substantially inactive in the presence of wild-type p53 but is active in the absence of wild-type p53.

Thus, the topoisomerase II α promoter is expressed in proliferating cells (including tumour cells) but since proliferating cells in the body (other than tumour cells) have wild-type p53, the topoisomerase II α promoter in the genetic construct of the invention is under negative regulation in these cells.

Topoisomerase II α is expressed in all normal proliferating cells; however, specificity arises from the higher proliferation index of the tumour cells coupled with a higher level of topoisomerase II α promoter activity in the absence of wild-type p53.

It will be appreciated that some variants of the topoisomerase II α promoter in which one or more base pair changes or deletions have been made will

also work in the invention, and that others will not.

For example, it has been shown that mutation of the inverted CCAAT box number 2 (ICB2 in Figure 8) from ATTGG to ATTCC abolishes the down
5 regulation of promoter activity that normally occurs when cells stop proliferating. Thus, a promoter carrying this mutation would express in both proliferating tumour cells and non-proliferating normal cells.

The skilled person, by routine experimentation, can readily determine
10 which mutations are useful and which are not. For example, the usefulness of a particular mutant promoter can be determined by linking the mutant promoter to a reporter gene, such as the chloramphenicol acetyl transferase (CAT) gene, or, indeed the topoisomerase II α gene itself and studying its expression in particular tumour and normal cells at
15 different stages of the cell cycle (see Isaacs *et al* (1996) *J. Biol. Chem.* **271**, 16741-16747; Hochhauser *et al* (1992) *J. Biol. Chem.* **267**, 18961-18965; and Sandri *et al* (1996) *Nucl. Acids Res.* **24**, 4464-4470).

Thus, in this embodiment the invention includes the use of a human DNA
20 topoisomerase II α promoter element to differentially express therapeutic genes in tumour cells which lack wild-type p53.

The results presented in Example 1 define a 101 bp region of the topoisomerase II α (topo II α) promoter which is negatively regulated by
25 wild-type p53, but not by mutant p53. The p53 gene is mutated or deleted in over 50% of human malignancies. Thus expression from the topo II α promoter element will not be repressed in the cells of many human tumours (this may be a reason for the differential sensitivity of tumour cells to topoisomerase II-targeting drugs). In addition, expression from
30 the topo II α promoter is elevated in cells which are rapidly dividing. This

provides another level of selectivity of expression since tumour cells are highly proliferative.

In this embodiment the topo II α promoter element drives the expression of a therapeutic gene such that it was expressed at higher levels in tumour cells (lacking wild-type p53) than in normal tissues (containing wild-type p53). The therapeutic gene could encode, for example, an enzyme capable of activating an inert form of a drug to a form which will kill the cell. Thus cell killing would selectively occur in the tumour cells where the enzyme was present at higher levels than in normal cells. As discussed in more detail below additional elements conferring tissue specificity and/or enhanced expression levels could be incorporated into the vehicle used to deliver the therapeutic gene. The topo II α promoter element could thus be generally useful in cancer gene therapy.

15

It is also preferred if the tumour suppressor gene is the *Rb* (retinoblastoma) gene. The retinoblastoma susceptibility gene product, pRb, acts as a transcriptional inhibitor and mutation or deletion of pRb (which occurs in at least some tumour cells) derepresses gene expression.

In this case, the mechanism appears to be the release from sequestration (by pRb) of a transcription factor that then activates gene expression. Rb loss of function is particularly associated with retinoblastomas, osteosarcomas, SCLCs, prostate and breast cancers.

Promoters repressed by Rb and therefore useful in the practice of the invention include those from the genes for *c-myc*, *cdc2* and IL-6 (see, for example, Salcedo *et al* (1995) *Arch. Med. Res.* **26**, 5157-5162; Yu *et al* (1992) *J. Biol. Chem.* **267**, 10203-10206; Hamel *et al* (1992) *Mol. Cell. Biol.* **12**, 3431-3438; Dalton (1992) *EMBO J.* **11**, 1797-1804; and Santhanam *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7605-7609.

Suitably, the genetic construct of the invention (whether it contains a topoisomerase II α -derived promoter element or some other suitable promoter element as defined) further comprises a genetic element which generally enhance the expression of the cytotoxic gene, at least once its
5 expression has been activated in the tumour cell.

Suitable enhancers include viral enhancers such as those from SV40, CMV or retroviral LTRs which would generally enhance expression of the cytotoxic gene. For example, the enhancers described by Toloza *et al.*
10 (1996) *Cancer Gene Therapy* 3, 11-17; Hoganson *et al* (1996) *Cancer Res.* 56, 1315-1323; Migita *et al* (1995) *Proc. Natl. Acad. Sci. USA* 92, 12075-12079; Marms & Splitter (1995) *Hum. Gene Ther.* 6, 1291-1297; Tani *et al* (1995) *Leukemia* 9, Suppl 1, 564-565; Xu *et al* (1995) *Gene* 160, 283-286; Tsan *et al* (1995) *Am. J. Physiol.* 268, L1052-L1056; and Balland
15 *et al* (1995) *Am. J. Physiol.* 268, L839-L845 may be useful.

Conveniently, the genetic construct of the invention (whether it contains a topoisomerase II α -derived promoter element or some other suitable promoter element as defined) further comprises a genetic element which
20 confers tissue-selective or cell-type-selective expression, or further comprises a genetic element which confers further tumour-selectivity.

Useful genetic elements are given below but new ones are being discovered all of the time which will be useful in this embodiment of the
25 invention.

The tyrosinase and TRP-1 genes both encode proteins which play key roles in the synthesis of the pigment melanin, a specific product of melanocytic cells. The 5' ends of the tyrosinase and tyrosinase-related
30 protein (TRP-1) genes confer tissue specificity of expression on genes

cloned downstream of these promoter elements.

The 5' sequences of these genes are described in Bradl, M. *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 164-168 and Jackson, I.J. *et al* (1991) *Nucleic Acids Res.* **19**, 3799-3804.

Prostate-specific antigen (PSA) is one of the major protein constituents of the human prostate secretion. It has become a useful marker for the detection and monitoring of prostate cancer. The gene encoding PSA and its promoter region which directs the prostate-specific expression of PSA have been described (Lundwall (1989) *Biochem. Biophys. Res. Comm.* **161**, 1151-1159; Riegman *et al* (1989) *Biochem. Biophys. Res. Comm.* **159**, 95-102; Brawer (1991) *Acta Oncol.* **30**, 161-168).

Carcinoembryonic antigen (CEA) is a widely used tumour marker, especially in the surveillance of colonic cancer patients. Although CEA is also present in some normal tissues, it is apparently expressed at higher levels in tumorous tissues than in corresponding normal tissues. The complete gene encoding CEA has been cloned and its promoter region analysed. A CEA gene promoter construct, containing approximately 400 nucleotides upstream from the translational start, showed nine times higher activity in the adenocarcinoma cell line SW303, compared with the HeLa cell line. This indicates that *cis*-acting sequences which convey cell type specific expression are contained within this region (Schrewe *et al* (1990) *Mol. Cell. Biol.* **10**, 2738-2748).

The mucin gene, MUC1, contains 5' flanking sequences which are able to direct expression selectively in breast and pancreatic cell lines, but not in non-epithelial cell lines as taught in WO 91/09867.

The alpha-fetoprotein (AFP) enhancer may be useful to drive pancreatic tumour-selective expression (Su *et al* (1996) *Hum. Gene Ther.* 7, 463-470).

- 5 The genetic constructs of the invention can be prepared using methods well known in the art.

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance,
10 complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

- 15 Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini
20 with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

- The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large
25 molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme
30 and ligated to an expression vector that has been cleaved with an enzyme

that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International
5 Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

10

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into
15 expression vectors using methods known in the art.

The present invention also relates to a host cell transformed with a genetic (preferably DNA construct) construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred
20 prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and
25 mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells
30 available from the ATCC as CCL61. NIH Swiss mouse embryo cells

NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650.

Transformation of appropriate cell hosts with a DNA construct of the
5 present invention is accomplished by well known methods that typically
depend on the type of vector used. With regard to transformation of
prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl.
Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning,
A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring
10 Harbor, NY. Transformation of yeast cells is described in Sherman *et al*
(1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring
Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also
useful. With regard to vertebrate cells, reagents useful in transfecting
such cells, for example calcium phosphate and DEAE-dextran or liposome
15 formulations, are available from Stratagene Cloning Systems, or Life
Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in
the art for transforming yeast cell, bacterial cells and vertebrate cells.

20 For example, many bacterial species may be transformed by the methods
described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646
incorporated herein by reference. The greatest number of transformants
is consistently recovered following electroporation of the DNA-cell
25 mixture suspended in 2.5X PEB using 6250V per cm at 25 μ FD.

Methods for transformation of yeast by electroporation are disclosed in
Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

30 Successfully transformed cells, ie cells that contain a DNA construct of

the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the cytotoxic gene product as defined in the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

When the genetic construct is a plasmid DNA construct it can be purified. The DNA construct of the invention is purified from the host cell using well known methods.

For example, plasmid vector DNA can be prepared on a large scale from cleaved lysates by banding in a CsCl gradient according to the methods of Clewell & Helinski (1970) *Biochemistry* 9, 4428-4440 and Clewell (1972) *J. Bacteriol.* 110, 667-676. Plasmid DNA extracted in this way can be

freed from CsCl by dialyse against sterile, pyrogen-free buffer through Visking tubing or by size-exclusion chromatography.

Alternatively, plasmid DNA may be purified from cleared lysates using
5 ion-exchange chromatography, for example those supplied by Qiagen. Hydroxyapatite column chromatography may also be used.

It will be appreciated that the genetic construct; composition comprising the genetic construct and means for introducing said genetic construct into
10 a cell; and virus or virus-like particle comprising a genetic construct of the invention can all be used to treat a cancer patient.

Thus, further aspects of the invention provide the said genetic construct; composition and virus or virus-like particle for use in medicine.

15

The invention also provides a pharmaceutical composition comprising a genetic construct as defined; or a composition comprising the said genetic construct and means for introducing said genetic construct into a cell; or said virus or virus-like particle and a pharmaceutically acceptable carrier.
20 The carrier or carriers must be "acceptable" in the sense of being compatible with the genetic construct or composition or virus or virus-like particle of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

25

A further aspect of the invention provides a method of treating a host with a cancer, or a host which host may develop cancer, the method comprising administering to said host a therapeutically effective amount of a genetic construct of the invention or a virus or virus-like particle of the invention
30 or a composition comprising a genetic construct of the invention and

means for introducing said genetic construct into a cell.

Preferably, the host is a mammal with a cancer; more preferably the host is a human patient with a cancer.

5

Suitably, the tumour suppressor gene status of the host (more particularly of the tumour to be treated) is determined either prior to or during treatment to determine that the tumour suppressor gene has been mutated in a relevant place in the gene or deleted. This can be done by methods
10 known in the art such as PCR/sequencing or immunohistochemistry.

By "relevant place" we include those parts of the tumour suppressor gene which encode a portion of the tumour suppressor gene product which is involved, directly or indirectly, in the control of transcription from the
15 said promoter.

In this case, the appropriate genetic construct can be chosen for administration to a tumour type in which it will be maximally effective. In other words, if, for example, the tumour is shown to express mutant
20 p53 or p53 is absent, the genetic construct comprises a promoter element responsive to p53 in the said manner.

It will be appreciated that it is preferable to administer the said genetic construct or said virus or virus-like particle or said composition locally at
25 the site of the tumour or into the vasculature of the tumour. The vascularisation of many tumours, and the high permeability of tumour vasculature, allows efficient delivery to tumours *via* this route.

It will however be appreciated that the said genetic construct or said virus
30 or virus-like particle or said composition may be administered

systemically.

When the cytotoxic gene product is directly cytotoxic it may be sufficient to administer said genetic construct or said virus or virus-like particle or
5 said composition alone. However, as discussed below, it may be desirable to use the therapeutic method of the present invention with other forms of therapy.

When the cytotoxic gene product is indirectly cytotoxic it is preferable that
10 the said genetic construct or said virus or virus-like particle or said composition is administered to the host and that any additional component to make the method cytotoxic is also administered to the host. Preferably the additional component (such as a prodrug activated by an enzyme which is the indirectly cytotoxic gene product) is administered after the said
15 genetic construct or said virus or virus-like particle or said composition.

In a particularly preferred embodiment the invention provides a method of treating a mammal harbouring a tumour. Suitably, the mammal is first prepared for tumour therapy by administering a said genetic construct or
20 said virus or virus-like particle or said composition of the invention and allowing the genetic material therein to localise to and be expressed in the tumour. Suitably, the indirectly cytotoxic gene encodes an enzyme which is able to convert a relatively non-toxic prodrug into a cytotoxic drug.

25 The expression of said enzyme in the tumour can, if desirable, be measured in the tumour using methods well known in the art. The method then further comprises administering to the mammal a component (preferably a prodrug) which is acted upon by the indirectly cytotoxic gene product (preferably an enzyme which converts said prodrug to a cytotoxic
30 drug).

The said genetic constructs or said virus or virus-like particles or said compositions (and also any additional component such as a prodrug) are administered in any suitable way, usually parenterally, for example intravenously, intraperitoneally or intra-vesically, in standard sterile, non-pyrogenic formulations of diluents and carriers, for example isotonic saline (when administered intravenously).

The invention also provides a therapeutic system comprising a said genetic construct or a said virus or virus-like particle or a said composition wherein said cytotoxic gene is indirectly cytotoxic and an additional component acted upon by said indirectly cytotoxic gene or its product and which becomes cytotoxic by said action.

Preferably, the indirectly cytotoxic gene is cytosine deaminase or thymidine kinase and the said additional component is 5-fluorocytosine or ganciclovir, respectively.

The invention also provides a therapeutic system comprising a said genetic construct or a said virus or virus-like particle or a said composition and means for determining the tumour gene status of a tumour in a patient. Suitably, the said means include reagents for detecting mutations in tumour suppressor genes. Such means and reagents are well known in the art and include PCR primers, restriction enzymes, oligonucleotide probes and the like. Mutants of p53 can also be detected using antibodies.

Although current ethical practice does not allow germ line gene therapy, one embodiment of the invention provides for the introduction of a genetic construct of the invention into the germ line. The genetic construct would lay "dormant" (ie without expressing substantially any cytotoxic gene product) in normal, non-cancer cells. The cytotoxic gene would be

expressed to a cytotoxic level in those cells where a mutation or deletion of the tumour suppressor gene occurred (as when the cell becomes cancerous or more virulently cancerous). Thus, the system may be used as a monitor of, and destroyer of cancer cells, in mammals, especially
5 humans, who are healthy individuals. It will be appreciated that when the cytotoxic gene is an indirectly cytotoxic gene that the additional component (such as a prodrug when appropriate) is administered periodically to "sweep out" (ie kill) the tumour cells.

10 Notwithstanding the above embodiment, the preferred method is to treat a host which host has a cancer.

The methods of the invention can be used to treat any cancer in which mutation of a tumour suppressor gene is implicated. As stated above, p53
15 is mutated in at least 50% of all human cancers.

The method is particularly suitable for:

(a) Tumour types displaying a high frequency of p53 mutations (these
20 are generally not as responsive to currently available therapies as tumours that rarely harbour p53 mutations). Examples of types displaying a high frequency of p53 mutations include lung, colon, head and neck, lymphoma, pancreas and stomach. Breast and prostate have a lower frequency (20-25%) but are common cancers.

25
(b) Tumour types expressing high levels of topoisomerase II α (when the promoter of the genetic construct used is the topoisomerase II α promoter). These include Hodgkin's disease, high grade lymphomas, squamous non-small cell lung cancers, seminomas and
30 drug resistant colon tumours.

Tumour types with both these attributes would be particularly suitable.

It is particularly preferred if the method is used to treat cancers in which the p53 tumour suppressor gene is mutated or deleted. It is particularly preferred to use a genetic construct wherein the element comprises the human topoisomerase II α promoter or a portion thereof.

Tumours with a high growth fraction ie contain a large proportion which are actively dividing, are associated with a much worse prognosis in general. The genetic construct comprising the human topoisomerase II α promoter or a portion thereof is particularly preferred for the treatment of such tumours.

Preferably, before starting treatment (or possibly during the course of treatment) an analysis of the growth fraction of a particular tumour is performed. This can be done using methods well known in the art such as by immunohistology using antibodies to cell proliferation antigens such as Ki-67 or proliferating cell nuclear antigen (PCNA) (see, for example, Haerslev *et al* (1996) *Breast Cancer Res. Treat.* 37, 101-113). It is preferred if the treatment is used on a tumour with a high proliferation index. Additionally or alternatively, immunohistology using antibodies to topoisomerase II α is performed. In the case of a genetic construct wherein the element comprises the human topoisomerase II α promoter or portion thereof this would give some indication of the expected level of expression of the therapeutic gene in the tumour cells.

Preferably, the p53 status of the tumour (ie determination of whether the p53 gene has been mutated or deleted) is carried out using methods well known in the art, for example by PCR/sequencing or by immunohistochemistry.

Preferred mutations are those that relieve repression of the topoisomerase II α promoter.

The tumour types where p53 is mutated frequently are given above; thus,
5 it may not be necessary to determine the p53 status in many cases. However, the method of treatment may include determination of the p53 status, and in particular the presence of particular p53 mutations, before or during treatment.

10 Cytotoxic chemo- or radio-therapy is known to upregulate wild-type p53 in normal tissues thereby inhibiting expression of the cytotoxic gene product from the genetic construct. Thus, it is preferred to combine the gene therapy method of the present invention (whether the said tumour suppressor gene is p53 or not) with the administration of a
15 chemotherapeutic agent to the host and/or with the administration of radiotherapy to said host.

Cancer chemotherapeutic agents include: alkylating agents including nitrogen mustards such as mechlorethamine (HN₂), cyclophosphamide,
20 ifosfamide, melphalan (L-sarcolysin) and chlorambucil; ethylenimines and methylmelamines such as hexamethylmelamine, thiotepa; alkyl sulphonates such as busulfan; nitrosoureas such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU) and streptozocin (streptozotocin); and triazenes such as decarbazine (DTIC; dimethyltriazenoimidazole-
25 carboxamide); Antimetabolites including folic acid analogues such as methotrexate (amethopterin); pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine
30 (6-thioguanine; TG) and pentostatin (2'-deoxycoformycin). Natural

Products including vinca alkaloids such as vinblastine (VLB) and vincristine; epipodophyllotoxins such as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorubicin (daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin C); enzymes such as L-asparaginase; and biological response modifiers such as interferon alphenomes. Miscellaneous agents including platinum coordination complexes such as cisplatin (*cis*-DDP) and carboplatin; anthracenedione such as mitoxantrone and anthracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N-methylhydrazine, MIH); and adrenocortical suppressant such as mitotane (*o,p'*-DDD) and aminogluthethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen.

When the genetic construct is one which comprises an element responsive to the p53 gene product it is desirable that the cytotoxic gene is indirectly cytotoxic and that its product activates a prodrug. Any cytotoxic drug that was released from the prodrug and which was available to normal cells which contain wild-type p53 will activate p53 expression and switch off expression of the cytotoxic gene from the genetic construct that may have been taken up by the normal cells. This should limit the overall cytotoxic effect in adjacent normal tissue.

The topoisomerase II α gene promoter may be used in a dual control unit. Thus, a preferred embodiment of the invention includes a first genetic construct comprising a topoisomerase II α gene promoter and a cytotoxic gene and (either as part of the first genetic construct or as a separate genetic construct) a second genetic construct in which expression of a second gene for down-regulating the cytotoxic gene in non-tumour cells is controlled by a second promoter that is up-regulated in non-tumour

cells. This type of dual control unit system is described in WO 97/12970 incorporated herein by reference.

The dual construct strategy exploits differences in the transcriptional
5 activation and stability functions of the p53 protein between normal and
tumour cells. More particularly, it utilises the dual transcriptional
regulatory function of wild-type p53 (wtp53) to eliminate or minimise
expression of the antitumour gene in normal cells and to enhance its
expression in tumour cells. For this purpose, the strategy proposes a
10 combination of two genetic units. In genetic unit 1 (the topoisomerase II α
promoter linked to a cytotoxic gene), the cytotoxic gene is controlled by
a promoter whose function is suppressed by wtp53, but is not suppressed
by mutant p53 (mp53), indeed it may even be up-regulated by mp53. In
genetic unit 2 (as is described above as the second genetic construct), a
15 gene for down-regulating the cytotoxic gene in normal cells is controlled
by a promoter containing the p53 binding site, and which is therefore
potently up-regulated by wtp53 but not by mp53. In other words, the
wtp53 in normal cells is used to up-regulate a gene which downregulates
the expression of the cytotoxic agent in those normal cells. The down-
20 regulating gene may for example express antisense RNA to the cytotoxic
gene, or a specific ribozyme for cytotoxic gene RNA, or a specific
transcription suppressor for the cytotoxic gene. It will be appreciated that
the p53 tumour suppressor gene product is believed to control at least in
part the expression of the cytotoxic gene; however, the dual construct
25 strategy may be used in an analogous fashion with respect to any tumour
suppressor gene product which is able to control at least in part the
expression of the cytotoxic gene.

Thus, this embodiment of the invention is preferably based on the p53
30 gene, because of its central role in tumour suppression and the differences-

in its function in normal and tumour cells. However, the general concept of this aspect of the invention may be applicable using other genes to up-regulate the cytotoxic agent in tumour cells and to down-regulate it in normal cells. Since the natural tumour suppressor pathways are complex and involve many genes, at least some of them could be used in a similar fashion.

Constructs for use in this embodiment of the invention comprise two separate genetic units. The cytotoxic gene is driven by a topoisomerase II α promoter ("type I") promoter which is not suppressed by, and may even be stimulated by, mutant p53 but is suppressed by wild-type p53. The second genetic unit is designed to suppress any leaky expression of the cytotoxic gene in normal cells. The promoter for this unit (type II promoter) typically contains the p53 consensus binding sequence, which is only activated by wild-type p53, within the context of a minimal promoter comprising a transcriptional start site and TATA Box. The p53 binding sequence may be included upstream of the minimal promoter or downstream. It may be useful if the p53 binding sequence is downstream from the promoter in genetic unit 2.

20

Apart from p53, other genes associated with the tumour suppression pathway may be utilised in the present invention.

Various means of suppressing leaky transcription in normal cells may be employed and three different approaches may be considered:

- (i) one approach employs an antisense construct (eg for the cytotoxic gene) driven by the type II promoter (eg p53 responsive). No antisense transcript should therefore be produced in tumours harbouring only mutant p53, but this construct should be transcriptionally activated within normal

30

cells and lead to down-regulation of any functional cytotoxic gene transcript driven by the type I promoter. It should be noted that even though normal cells express only very low levels of wild-type p53, the introduction of gene constructs by transfection has been shown to induce
5 expression of detectable wild-type p53. Such induction may therefore help to improve discrimination between normal and tumour cells. Low dose radiation, which would be expected to induce wt p53 in normal but not tumour cells, may also improve discrimination.

10 (ii) Another approach to suppressing the level of activity of a cytotoxic gene in normal cells involves the use of a specific ribozyme designed to complex with and cleave the target (cytotoxic gene) mRNA.

(iii) A third approach uses a sequence-specific transcriptional
15 suppressor. Several such transcriptional suppressors have been characterised. Experiments may include use of the binding domain of the yeast Gal-4 transcription factor and the suppression domain of the *Drosophila* even-skipped protein under the control of the type II promoter to suppress expression by means of a tandem repeat of the Gal-4 target
20 sequence cloned into the type I promoter.

A combination of two or more such approaches may be utilised. However, the most appropriate approach may vary according to the cytotoxic gene being expressed.

25

If antisense or ribozyme technology is employed, the sequence expressed by the Type II unit must be tailored to the sequence expressed by the Type I unit, adding a level of complexity to experimentation. In contrast, the use of a transcriptional suppressor has the advantage of allowing the
30 construction of a "universal cassette" into which different coding

sequences, eg different cytotoxic genes, may be inserted under the control of the Type I promoter without there being a need for tailoring of the Type II unit.

- 5 In experimental optimisation of the antitumour system of this embodiment the present invention, initially the type I and type II promoter constructs may be tested individually, to assess the magnitude of the effects which can be generated. Co-transfections may then be carried out to assess the combined effects of both constructs in determining overall expression
10 levels of a reporter gene such as luciferase in the presence of wild-type or mutant p53. Finally, both genetic units may be incorporated into a single construct in either a head-to-head, tail-to-tail or head-to-tail arrangement, in the latter case having the type II promoter upstream of the type I promoter, or *vice versa*. If it is observed that there is transcriptional or
15 enhancer interference between the two constructs, this may be overcome by altering the relative distance between and/or orientation of the units, and/or by linearisation of the construct, and/or by the introduction of so-called interrupter (insulator) sequences, which have been characterised for example in *Drosophila* and chicken systems, and shown to prevent
20 interactions between enhancer elements in adjacent transcription units. Further details of these features are described in WO 97/12970 and are incorporated herein by reference.

The invention will now be described in more detail with reference to the
25 following Examples and Figures in which:

Figure 1 shows the concentration-dependent inhibition of human topoisomerase II α promoter activity by wild-type p53. Different concentrations of wild-type p53 expressing plasmid were co-transfected
30 with 10 μ g of topoisomerase II α -CAT construct in SKOV3 cells. After

48 hours, cells were lysed and a CAT assay was performed on the extract.

Figure 2 shows the topoisomerase II α promoter activity in cells expressing no p53 (SKN), mutant p53 (SK23 at 37°C) or wild-type p53 (SK23 at 32°C). In each case, the transfected cells were divided between two dishes, one of which was grown at 32°C and the other at 37°C. After 48 hours, cells were lysed and a CAT assay was performed on the extract. Three independent assays of CAT activity on extracts from SKN cells and two independent assays on extracts from SK23 cells are shown.

10

Figure 3 shows a Western blot analysis showing transcriptional activation of p21 upon shift down of Saos-2 ts p53 cells to 32°C. Proteins were transferred to a nitrocellulose membrane and probed with antibodies to both p53 and p21. The positions of p53 and p21 are indicated on the right. Note the presence of p21 in the sample from cells grown at 32°C, but not those grown at 37°C.

15

Figure 4a is a diagrammatic representation of the promoter constructs employed. The 5' limit of each promoter fragment is indicated on the left. The position of the GC boxes (GC), inverted CCAAT boxes (ICB), ATF site (ATF) and growth hormone reporter gene (HGH) are indicated. The major transcription start site is marked with an arrow.

20

Figure 4b shows the percentage of growth hormone (HGH) expression 120 h after transfection of Saos-2 ts p53 cells with various different lengths of the topoisomerase II α promoter (indicated on the X axis). In each case, the transfected cells were divided between two dishes, one of which was grown at 32°C and the other at 37°C. The shaded bars represent the expression when the cells were cultured at 37°C (p53 mutant) and the black bars represent cells at 32°C (p53 wt). In each case, the level of

25

30

growth hormone at 37°C has arbitrarily been given a value of 100%.

Figure 4c shows the percentage of growth hormone (HGH) expression as a function of cell number 120 h after transfection of Saos-2 ts p53 cells with 144 bp and 101 bp topoisomerase II α promoter constructs. The shaded bars represent those cells cultured at 37°C (p53 mutant) and the black bars represent cells grown at 32°C (p53 wt).

Figure 5 shows the percentage of growth hormone expression (HGH) 120 h following transfection of Saos-2 cells with mutated forms of the topoisomerase II α promoter (ICB1 and GC1) alone (shaded bars), or following co-transfection with the wild-type p53-expressing construct pLSVhp53c62 (black bars). The HGH expression level in the absence of p53 was arbitrarily given a value of 100%.

Figure 6 shows the cell cycle distribution of Saos-2 ts p53 cells transfected with the topoisomerase II α 101 bp promoter construct and the control promoters PGKGH (fusion construct of human phosphoglycerate kinase 1 promoter and human growth hormone) and FGH (fusion construct of mouse ferritin heavy subunit promoter and human growth hormone). The cells were cotransfected with CD2 cell surface marker and the cell cycle distribution of the CD2-positive transfectants was analysed 120 hours after transfection. The upper traces represent cells cultured at 37°C and the lower black traces cells cultured at 32°C.

Figure 7 shows the percentage of growth hormone expression (HGH) 120 h after transfection of Saos-2 ts p53 cells with the control promoters PGKGH and FGH. The shaded bars represent expression of HGH when the cells were cultured at 37°C (mutant p53) and the black bars cells cultured at 32°C (wt p53). The expression level of HGH at 37°C in each

case was arbitrarily given a value of 100%.

Figure 8 shows *a*, sequence of the human topoisomerase II α promoter. Bases are numbered (as indicated on the left) with respect to the major transcription start site, which is designated +1 and marked with an arrow. Putative *cis*-acting elements, underlined and annotated above, include GC boxes (Sp1 sites) at position -562 and -51, ICBs at positions -385, -259, -175, -108, and -68, and an ATF site at -226. The ATG start codon is overlined. *b*, schematic representation of putative transcription factor binding motifs and the truncated promoter derivatives constructed. The 5' limit of each promoter fragment is indicated on the left. The major transcription start site is marked with an arrow. The positions of the GC boxes (GC), ICBs (C), ATF site (ATF), and growth hormone reporter gene (HGH) are indicated.

Figure 9 shows that the CRB and 3H10 antisera detect 170 kDa and 180 kDa proteins, respectively, in human cell nuclear extracts. A 0.35 M NaCl nuclear protein extract prepared from NCI460 cells (lanes 2 and 4) or NCI460/pV8 cells (lanes 1 and 3) was electrophoresed alongside molecular weight standards on a 9% SDS-polyacrylamide gel, transferred to Hybond-N, and the membrane was exposed either to the CRB antiserum (Lanes 1 and 2) or to a mixture of the CRB and 3H10 antisera (Lanes 3 and 4). Antibody detection was the ECL system. Molecular weights (in kDa) are indicated on the left. The positions of the 170 kDa topoisomerase II α and the 180 kDa topoisomerase II β protein are indicated on the right.

Figure 10 shows the staining of tissues for topoisomerase II α and β . Left hand side pictures are stained for topoisomerase α (Figures A, C, E and G). Right hand side pictures are stained for topoisomerase β (B, D, F,

H).

A and B show tonsil sections at low power and C and D are tonsil sections at higher power. Topoisomerase α (A & C) is mainly restricted to the larger cells in the germinal centre (centroblasts), whereas topoisomerase β (B & D) is very widely distributed in all cell types, including the B and T cell areas.

E and F show sections of a squamous cell carcinoma of the lung and G and H show a case of Hodgkin's disease. The distribution of topoisomerase II α staining is similar to that seen with anti-proliferation-associated antibodies (such as Ki-67), whereas topoisomerase β is found in the majority of cell types, including Reed-Sternberg cells in Hodgkin's disease (Figure H).

15

Figure 11 shows the expression of topoisomerase II α and β in cytospin preparations of the SUDHL-1 cell line using immunoperoxidase staining. Panels: (A and C), localisation of topoisomerase β in SUDHL-1 stained with antibody 3H10. The antigen is present as granular spots in the interphase nucleus, but during mitosis the antigen is absent from the nucleus and present as spots in the cytoplasm. (B and D), localisation of topoisomerase II α using the CRB antibody. The antigen is present on the whole of the nucleus with stronger staining in nucleolar areas (cells in mitosis show antigen associated with condensed chromatin, not in the cytoplasm).

20

25

Example 1: p53 regulates the minimal promoter of the human topoisomerase II α gene

SUMMARY

5

DNA topoisomerase II α is an essential enzyme for chromosome segregation during mitosis. Consistent with a cell division-specific role, the expression of the topoisomerase II α gene is strongly influenced by the proliferation status of cells. The p53 protein is one of the most important regulators of cell cycle progression in mammals, with an apparent dual role in the induction of cell cycle arrest following cytotoxic insults and in the regulation of the apoptotic cell death pathway. We have analysed whether p53 plays a role in regulating expression of the human topoisomerase II α gene. We show that wild-type, but not mutant, p53 is able to decrease substantially the activity of the full length topoisomerase II α gene promoter. Using a series of constructs comprising various deleted or mutated versions of the promoter lacking critical *cis*-acting elements, we show that this p53-specific regulation of the topoisomerase II α promoter is independent of all characterised transcription factor binding sites and is directed at the minimal gene promoter. We conclude that expression of wild-type p53 induces down-regulation of the human topoisomerase II α promoter by acting on the basal transcription machinery. These findings implicate topoisomerase II α as one of the downstream targets for p53-dependent regulation of cell cycle progression in human cells.

10

15

20

25

RESULTS

Wild-type p53 negatively regulates the topoisomerase II α gene promoter

- 5 In order to determine whether p53 has a role in regulating expression of the topoisomerase II α gene, we analysed the ability of wild-type p53, expressed from pLSVhp53c62, to modulate expression of a CAT reporter gene that was linked to the full-length, 2.5 kb topoisomerase II α gene promoter (Hochhauser *et al*, 1992). Following transient transfection of
- 1 SKOV3 cells with different concentrations of the human wild-type p53 expression construct, together with a constant amount of the full length topoisomerase II α promoter/CAT plasmid, a concentration-dependent repression of promoter activity was observed. An approximately 90% reduction in CAT activity was found in the presence of 15 μ g of the wt
- 5 p53 expression construct (Figure 1).

- In the same SKOV3 cell line, we also tested whether the observed repression of topoisomerase II α promoter activity was specific for wild-type p53. Table 1 shows the percentage inhibition of CAT expression
- 1 from the 2.5 kb promoter fragment brought about by co-expression of wild-type human or murine p53, and by two mutated derivatives of p53. Whilst co-expression of wild-type p53 induced a 70-90% inhibition of CAT activity, only a modest down-regulation of CAT activity was seen in cells co-expressing the mutant p53 proteins. These results suggested
- 5 that the down-regulation of topoisomerase II α promoter activity was relatively specific for wild type p53.

Table 1: Percentage inhibition of CAT expression from the 2.5 kb fragment of topoisomerase II α promoter by wild-type or mutant p53. The vector only control was arbitrarily given a value of 100%.

5	Constructs Used	% CAT Activity
	vector alone	100
	human wt	34 \pm 7
	human mutated (273 arg to his)	80 \pm 11
	murine wt	10 \pm 2
10	murine mutated (135 val to ala)	62 \pm 21

Down-regulation of Topoisomerase II α promoter activity is specific for wild-type p53

- 15 To confirm that wild-type, but not mutant, p53 was capable of down-regulating the topoisomerase II α gene promoter, we took advantage of an expression construct, designated pLTRp53cGVal 135, encoding a murine p53 protein that assumes a wild-type conformation at 32°C, but a mutant conformation at 37°C (Michalovitz *et al*, 1990). The construct containing
- 20 the CAT reporter gene under the control of the 2.5 kb fragment of the topoisomerase II α promoter was transfected into a clone of SKOV3 cells (designated SK23a), into which the construct encoding the temperature sensitive p53 had been stably integrated. Following transfection, the cells were cultured at either 32°C or 37°C, and the level of CAT expression
- 25 was quantified. A clone of SKOV3 cells stably transfected with the neomycin-containing vector alone (SKN) was studied in parallel as a control. Figure 2 shows that the temperature shift had little or no effect on expression of CAT in the control SKN cells. In contrast, while CAT activity was readily detectable in extracts of SK23a cells grown at 37°C,
- 30 a dramatic reduction in CAT activity was seen in the cells maintained at 32°C. Because a single population of cells was divided equally and

incubated at the different temperatures only after transfection, the effects of wild-type p53 on promoter activity could not be explained by differences in the ability of DNA constructs to transfect the host cell line. Thus, we conclude that p53 down-regulates expression from the topoisomerase II α gene promoter and that this effect is specific for p53 in its wild type conformation.

p53 acts on the minimal topoisomerase II α gene promoter

The results of the above experiments raised a number of questions: does p53 act through interaction with a specific binding sequence in the topoisomerase II α gene promoter, or could the observed effects of p53 be mediated via perturbations of cell growth, or cell cycle progression? If expression of p53 were to arrest the cells in the G0/G1 phase of the cell cycle, expression of the topoisomerase II α promoter might be affected, since expression of topoisomerase II α mRNA is regulated in a cell cycle phase-specific manner. To address these issues, and to confirm the results presented above, we utilised a p53^{null} human cell line, Saos-2, into which a CMV-based construct expressing a temperature sensitive human p53 (Val138-Ala) had been stably integrated (designated Saos-2 ts p53). As before, this p53 protein adopts a wild-type conformation at 32°C and a mutant conformation at 37°C. Immunohistochemical staining confirmed that the p53 protein was expressed in these cells at both temperatures, but that it localised to the nucleus in cells incubated at 32°C (data not shown). Moreover, the wild type p53 was shown to be functional since expression of the p53-regulated p21 protein (WAF1/CIP1) was seen in cells grown at 32°C but not at 37°C (Figure 3). For the analyses of Saos-2 ts p53 cells, we used a series of topoisomerase II α promoter constructs generated for analysis of the growth-state regulation of the promoter (Isaacs *et al*, 1996). Instead of the CAT gene, these constructs contain human growth

hormone (HGH) as a reporter gene cloned downstream of various different fragments of the human topoisomerase II α promoter. Figure 4a shows a diagrammatic representation of the different promoter constructs employed. Following transient transfection, the level of expression of HGH from constructs containing 617, 210, 144 and 101 bp of the promoter was in each case substantially lower in Saos-2 ts p53 cells cultured at 32°C, than in those cells cultured at 37°C (Figure 4b). To exclude effects of incubation temperature on cell cycle transit times, we measured cell numbers at the end of each experiment. HGH expression as a function of cell number still showed a substantially reduced level at 32°C as compared to 37°C (Figure 4c). Indeed, temperature shift did not dramatically affect cell proliferation over the time-course of these experiments, since cell numbers generally differed by no more than 50% in the transfected cell populations incubated at 32°C and 37°C. These data confirm those generated using CAT reporter constructs in the SKOV3 cell line, and indicate that the activity of the previously described minimal promoter of the topoisomerase II α gene (~ 100 bp of sequence upstream of the CAP site) is negatively regulated by p53.

The results presented thus far demonstrate that many of the *cis*-acting elements previously identified within the topoisomerase II α promoter, which lie in the region between -101 and -617 (Figure 4a), can be eliminated as candidates for the target of p53 action on promoter activity. However, the 100 bp minimal promoter does contain two well-defined consensus transcription factor binding elements, a GC box (a potential Sp1 binding site) at position -44, and an inverted CCAAT box (ICB1) at position -64 (see Figure 4a). To study whether these elements were implicated in the regulation of promoter activity by p53, we mutated the potential GC box and ICB1 elements in the 617 bp promoter/growth hormone fusion construct and cotransfected Saos-2 cells with these

mutated constructs and the wild-type p53 expressing construct, pLSVhp53c62. Figure 5 shows that mutation of these elements did not abrogate the down-regulation of promoter activity seen in Saos-2 cells when wild-type p53 was expressed.

5

p53 does not act via perturbing the cell cycle

We next addressed the possibility that the wild-type p53 protein was influencing the cell cycle distribution of the transfected cells and that this was indirectly affecting topoisomerase II α promoter activity. To study this, Saos-2 ts p53 cells were cotransfected with the pKV461/CD2 expression vector encoding the CD2 cell surface protein (to act as an antigenic tag for transfected cells) and the 101 bp topoisomerase II α promoter fragment linked to HGH. The control promoters, ferritin (FGH) and phosphoglycerate kinase (PGKGH), were also co-transfected with the pKV461/CD2 vector into Saos-2 ts p53 cells. Following transfection, the cells were cultured at either 32°C or 37°C. Figure 6 shows that the cell cycle distribution of the cells cultured at 32°C (when p53 would be wild type) and at 37°C (when p53 would be mutant) was very similar. Thus, cell cycle perturbations are unlikely to account for the p53-induced promoter down-regulation.

p53 acts specifically on the topoisomerase II α gene promoter

In order to eliminate the possibility that wild-type p53 was having a general negative influence over gene transcription in the transfected cells, we analyzed the effect of p53 on expression of the HGH reporter gene from two control promoters. Figure 7 shows that expression of HGH from the PGKGH and FGH gene promoters in Saos-2 ts p53 cells was not substantially down-regulated by temperature shift from 37°C to 32°C,

indicating that the activity of these promoters was not negatively regulated by co-expression of wild-type p53 protein. Indeed, the activity of the phosphoglycerate kinase gene promoter was somewhat higher in cells grown at 32°C than at 37°C.

5

DISCUSSION

We have shown that wild-type p53 is a negative regulator of the activity of the human topoisomerase II α promoter and that this effect is mediated through the minimal sequences required for topoisomerase II α promoter activity. This regulation is apparently independent of a perturbation in the cell cycle distribution of the transfected cells, and has been demonstrated in different cell lines using different constructs encoding either human or murine p53. Moreover, we have shown that p53 has some apparent specificity for the topoisomerase II α promoter, in that the activity of two control gene promoters was not down-regulated by expression of wild-type p53.

p53 interacts with DNA in a sequence-specific fashion binding to DNA containing two contiguous monomers of the sequence 5'-PuPuPuC (T/A) (T/A) GPyPyPy. These two elements are generally separated by between 0 and 13 bp of non-conserved sequence (El-Deiry *et al*, 1992). The topoisomerase II α promoter does not contain a precise match for this consensus p53 binding element. However, a similar sequence (AAGCTTTCCG-7 bp-AAACAAGTGA) is present between -269 and -295 bp of the topoisomerase II α promoter, but this motif contains two changes from the consensus in each unit of the p53 binding sequence (indicated above by underscoring). Consistent with this motif lacking any functional significance, we have been unable to detect any binding of wild-type p53 protein to oligonucleotide containing this sequence using gel retardation

assays (unpublished observations). Moreover, expression of the HGH reporter gene from a topoisomerase II α promoter construct lacking this element has been shown still to be negatively regulated by wild-type p53.

Because of the cell cycle regulatory role of p53, we addressed whether the topoisomerase II α promoter could be responding to cell cycle perturbation induced by p53, and not to a direct effect of p53 on the transcription machinery. No evidence was obtained for accumulation of cells in any particular cell cycle phase, in those cells in which wild-type p53 was expressed, at least over the time course of these experiments. Clearly, the other genetic changes that accompany the acquisition of a transformed state, such as alteration in the retinoblastoma susceptibility gene (which is not expressed in Saos-2 cells) or cyclin-dependent kinase inhibitors, could be influencing the efficiency with which wild-type p53 can mediate cell cycle arrest in the cell lines chosen for study here. We have shown elsewhere that the topoisomerase II α promoter responds to growth arrest signals (Isaacs *et al*, 1996). However, the regulatory elements in the promoter that respond to changes in growth state are distinct from those responding to p53, and are located upstream of the minimal topoisomerase II α gene promoter defined in this work. In particular, the 101 bp minimal promoter, which we have shown to be regulated by p53, lacks normal negative regulation brought about by inhibition of proliferation (Isaacs *et al*, 1996). Moreover, although topoisomerase II is a cell cycle regulated gene, recent data indicate that the variation in mRNA expression during the cell cycle is largely due to changes in transcript stability, not promoter activity (Goswami *et al*, 1996).

At least one action of p53 as a negative regulator of gene transcription appears to be directed towards components of the basal transcription machinery. An important feature of this effect may be the formation of

complexes between p53 and TATA box-binding protein (TBP)-associated factors, possibly TBP itself. Moreover, Liu and Berk (1995) have shown recently that p53 may act through direct or indirect interactions with both TFIIB and TFIID, which act as basal transcription factor complexes. Our data are consistent with the hypothesis that wild-type p53 acts via negatively regulating the basal transcription machinery required to effect expression of the topoisomerase II α gene. In other cases, such as in the regulation of SV40 and hsp70 gene transcription, p53 appears to disrupt the ability of a protein complex to bind to DNA that includes either the transcription factor Sp1 (GC box binding factor) (Perrem *et al.*, 1995), or CBF (CCAAT box binding factor) (Agoff *et al.*, 1993). Although consensus Sp1 and CCAAT boxes lie close to the CAP site in the topoisomerase II α gene promoter, mutation of these sites did not prevent p53 from negatively-regulating promoter activity. This suggests that Sp1 and CBF are unlikely to be important targets for p53 action in regulating the expression of the topoisomerase II α gene.

In summary, we have shown that wild-type p53 can specifically down-regulate the activity of the human topoisomerase II α gene promoter. The challenge is now to delineate the precise downstream consequences of this regulation and to ascertain whether topoisomerase II α gene expression is an important target for other regulators of cell cycle progression in human cells.

MATERIALS AND METHODS

Cells

The human ovarian cancer cell line SKOV3, which does not express p53 mRNA or protein (Yaginuma and Westphal, 1992), was grown in RPMI-

1640 medium supplemented with 10% foetal calf serum (FCS). The SK23a and SKN cell clones (Vikhanskaya *et al*, 1994) were both derived from SKOV3 cells. To generate the SK23a derivative, SKOV3 cells were co-transfected with a plasmid encoding murine temperature sensitive mutant p53 (Michalovitz *et al*, 1990) and the pSV2neo vector containing the neomycin selectable marker genes. The SKN cell line was derived by transfection of SKOV3 cells with the neomycin expression vector alone, and served as a negative control. The human osteosarcoma cell line Saos-2, which expresses no p53 (Diller *et al*, 1990) and no functional pRb protein (Huang *et al*, 1988), as well as a derivative (designated Saos-2 ts p53) that stably expresses a temperature sensitive human p53 protein due to a valine to alanine substitution at amino acid 138, were maintained in RPMI-1640 supplemented with 10% FCS. Where required for transfection using calcium phosphate (see below), cells were grown in Dulbecco's modified Eagles's Medium (DMEM) for 24 hours prior to addition of the precipitate. All cells were grown in a humidified atmosphere in the presence of 5% CO₂, and were regularly screened for the presence of mycoplasma.

General plasmid vectors

The construct encoding the wild-type human p53, designated pLSVhp53c62 (Zakut-Houri *et al*, 1985) utilises the SV40 early promoter in the vector pLSV. The construct for expression of mutant murine p53 (pLTRp53cGVal 135; kindly supplied by Dr M. Oren) has been described in detail elsewhere (Michalovitz *et al*, 1990), and encodes a temperature sensitive p53 that adopts a wild-type conformation at 32°C, but a mutant conformation at 37°C due to a mutation at position 135 (valine to alanine). The construct (designated pCMVtsp53Val 138) for expression of a human p53 that adopts a mutant conformation at 37°C and a wild-type

conformation at 32°C, was kindly provided by Dr J. Jenkins and is essentially identical to that described previously (Yamato *et al*, 1995). The vector for the expression of mutant human p53 (with an arginine to histidine mutation at position 273) contains the mutated cDNA under the control of the CMV promoter (kindly supplied by Dr P. Chumakov). Plasmids encoding the *E. coli* CAT gene under the control of different fragments of the human topoisomerase II α gene promoter, have been reported previously (Hochhauser *et al*, 1992). Plasmids carrying the mouse ferritin (FGH) and the human phosphoglycerate kinase 1 (PGKGH) gene promoters linked to the human growth hormone (HGH) coding region (Pugh *et al*, 1991; Firth *et al*, 1994) were kindly provided by Dr J. Firth (Oxford, UK). The pKV461/CD2 (kindly provided by Dr C.J. Norbury) contains a truncated rat CD2 cDNA and was generated by excising the CD2 cDNA from pERCD2-2 (He *et al*, 1988) and cloning it into the *Bgl*II site of pKV461 (kindly supplied by Dr M. Sowden; (Sowden *et al*, 1989).

Constructs encoding human growth hormone (HGH) from truncated or mutated versions of the human topoisomerase II α gene promoter

The plasmid used for all of the HGH constructs was PGEM7Zf+ (PROMEGA), containing the 1.8 kb HGH cDNA as a reporter gene. This plasmid, designated pSVGH, was a generous gift from Dr J. Firth, Oxford, UK. The SV40 promoter in pSVGH was replaced by the topoisomerase II α promoter to generate pHGH, as described previously (Isaacs *et al*, 1996). Deletion constructs containing various truncated forms of the topoisomerase II α promoter were generated by PCR from the full length 2.5 kb fragment of the promoter (Hochhauser *et al*, 1992). PCR primers incorporated restriction sites to enable directional cloning via the *Xba*I and *Hind*III, or the *Bam*HI sites in pHGH. Sequences for the 5'

primers (with the 5' limit indicated on the left) used in generating the promoter truncations were as follows. Numbering begins at +1, the transcription start site.

- 5 5' -GATCTCTAGAGCCACCGCACACAGCCTACTT-3'
 5' -GATCTCTAGATTGAAGCCTCTCTAGTCC-3'
 5' -GATCTCTAGAAGCCGTTTCATAGGTGGATAT-3'
 5' -GATCTCTAGACTTCTGGACGGAGACGGTGA-3'
 5' -GATCTCTAGAGCTTCGGGCGGGCT-3'

0

In each case, the 3' primer, which ran up to the translation start site, was as below:-

ATG 5' -GGATCAAGCTTATGGTGACGGTCGTGAAGG-3'

5

After directional cloning of the PCR products into pHGH, the orientation and sequence of all promoter fragments was confirmed.

Transfection of SKOV3 cells

)

Exponentially growing cells were transfected with 1-15 μ g of the p53 expression vectors and 10 μ g topoisomerase II α promoter/reporter gene constructs using the CaPO₄ co-precipitation procedure of Graham and van der Eb (1973). To achieve this, cells were grown in DMEM medium for 24 hours before transfection. 48 hours after transfection, cells were harvested and lysed by three successive cycles of freezing and thawing. An expression plasmid encoding the bacterial β -galactosidase gene was included in each transfection, both to monitor transfection efficiency and to standardize the amount of extract to be used in subsequent CAT assays. β -galactosidase assays were performed as described by Herbomel *et al* (1984). An equivalent concentration of DNA was used in all transfections by adjusting the level of control vector DNA.

Transfection of Saos-2 cells

Exponentially growing Saos-2 or Saos-2 ts p53 cells were cultured for 24 hours in DMEM before transfection by the CaPO_4 co-precipitation method of Graham and van der Eb (1973). Precipitates (1.5 ml), which contained 30 μg of the appropriate pGEM plasmid carrying the topoisomerase II α promoter linked to the HGH reporter gene, and, where required, 15 μg of pKV461/CD2 (O'Connell *et al*, 1994) or 15 μg of a control plasmid, were applied to subconfluent cells for 16 hours. The cells were then washed in phosphate buffered saline, trypsinized, and seeded at a ratio of 1:2 in RPMI-1640 medium. Cultures were then maintained either at 32°C or at 37°C for up to 120 hours.

Western blot analysis of Saos-2 ts p53 cells

Saos-2 ts p53 cells exponentially growing either at 37°C or after shift-down to 32°C were harvested and lysed directly in SDS-PAGE sample buffer. Equal quantities of soluble proteins were separated on a 12% SDS polyacrylamide gel and transferred overnight onto a nitrocellulose membrane (Hybond-ECL; Amersham). Proteins were detected using a mixture of the p53-specific monoclonal antibody DO-1 (Vojtesek *et al*, 1992) which detects both wild-type and mutant p53, and a p21^{CIP1/WAF1} specific monoclonal antibody (obtained from Upstate Biotechnology Incorporated). Immunoreactive proteins were detected using the enhanced chemiluminescence technique, as recommended by the supplier (Amersham).

CAT assays

CAT activity was measured by the conversion of ^{14}C -labelled chloramphenicol to its acetylated forms using standard techniques. The
5 % conversion was determined by excising radioactive spots from the thin layer chromatography plates and measuring the level of radioactivity in each sample in a scintillation counter.

HGH assays

Promoter activity was determined by measuring the level of the HGH gene product in the media of transfected cells in culture. The assay was conducted on aliquots of medium using an immuno-radiometric assay (IRMA) kit supplied by the North East Thames Radioimmune Assay (Netria) Co. This assay utilises a sheep polyclonal anti-HGH solid phase antibody and an ^{125}I -labelled murine monoclonal anti-HGH tracer antibody. All assays were kindly performed by Gillian Campling at Littlemore Hospital, Oxford, UK.

Site-directed mutagenesis

This was performed using the Muta-gene *in vitro* mutagenesis system (Bio-Rad), which utilizes oligonucleotide primers containing the appropriate point mutations, and a single-stranded template into which uracil residues have been incorporated to permit selection against the template strand of DNA. This protocol was based on the method of Kunkel *et al* (1987). The pHGH derivative containing the -617 promoter fragment was used to generate single-stranded DNA templates. Primers used for mutating the first inverted CCAAT box (ICB1) and the first GC box consensus (GC1) elements (see Figure 4a), were as follows:

Mutant GC1: 5' -GGTCTGCTTCGTGCGTGCTAAAGG-3'

Mutant ICB1: 5' -AGTCAGGGATTCCCTGGTCTGCTT-3'

DNA sequencing

5

Nucleotide sequencing was performed on double-stranded plasmid templates using the dideoxy chain termination method and Sequenase enzyme, as recommended by the suppliers (US Biochemical Corp.).

0 *Flow cytometry*

The detection of the CD2 cell surface antigen was achieved using a FITC-conjugated anti-rat CD2 monoclonal antibody (OX-34; SeroTec), as described by O'Connell *et al* (1994). Cells were fixed for 30 minutes in
5 ice-cold 70% ethanol in PBS, collected by centrifugation and were treated with RNase A (100 µg/ml final concentration) and propidium iodide (40 µg/ml) in PBS for 30 minutes at 37°C. Cell cycle distribution was then determined using a Becton-Dickinson FACScan, and the data were analyzed using Lysys II software.

0

REFERENCES FOR EXAMPLE 1

- Agoff *et al* (1993) *Science* **259**, 84-86.
- Diller *et al* (1990) *Mol. Cell. Biol.* **10**, 5772-5781.
- 5 El-Deiry *et al* (1992) *Nature Genetics* **1**, 45-49.
- Firth *et al* (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6496-6500.
- Goswami *et al* (1996) *Mol. Cell. Biol.* **16**, 1500-1508.
- Graham, F.L. and van der Eb, A.J. (1973) *Virology* **52**, 456-467.
- He *et al* (1988) *Cell* **54**, 979-984.
- 10 Herbolme *et al* (1984) *Cell* **39**, 653-662.
- Hochhauser *et al* (1992) *J. Biol. Chem.* **267**, 18961-18965.
- Huang *et al* (1988) *Science* **242**, 1563-1566.
- Isaacs *et al* (1996) *J. Biol. Chem.* **271**, 16741-16747
- Kunkel *et al* (1987) *Methods in Enzymol.* **154**, 367-382.
- 5 Liu, X. and Berk, A.J. (1995) *Mol. Cell. Biol.* **15**, 6474-6478.
- Michalovitz *et al* (1990) *Cell* **62**, 671-680.
- O'Connell *et al* (1994) *EMBO J.* **13**, 4926-4927.
- Perrem *et al* (1995) *Oncogene* **11**, 1299-1307.
- Pugh *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10553-10557.
- 1) Sowden *et al* (1989) *Nucl. Acids Res.* **17**, 2959-2972.
- Vikhanskaya *et al* (1994) *Nucl. Acids. Res.* **22**, 1012-1017.
- Vojtesek *et al* (1992) *J. Immunol. Meth.* **151**, 237-244.
- Yaginuma, Y. and Westphal, H. (1992) *Cancer Res.* **52**, 4196-4199.
- Yamato *et al* (1995) *Oncogene* **11**, 1-6.
- Zakut-Houri *et al* (1985) *EMBO J.* **4**, 1251-1255.

Example 2: The distribution and expression of the two isoforms of DNA topoisomerase II in normal and neoplastic human tissues

Summary

5

In mammalian cells, there are two isoforms of DNA topoisomerase II, designated α (170 kDa form) and β (180 kDa form). Previous studies using cell lines have shown that the topoisomerase II α and β isoforms are differentially regulated during the cell cycle and in response to changes in growth state. Moreover, both isoforms can act as targets for a range of antitumour drugs. Here, we have analysed the normal tissue distribution in humans of topoisomerase II α and β using isoform-specific antibodies. In addition, we have studied expression of these isoforms in 69 primary tumour biopsies, representative either of tumours that are responsive to topoisomerase II-targeting drugs (breast, lung, lymphoma and seminoma) or of those that show *de novo* drug resistance (colon). Topoisomerase II α was expressed exclusively in the proliferating compartments of all normal tissues, and was detectable in both the cell nucleus and cytoplasm. In biologically aggressive or rapidly proliferating tumours (eg high grade lymphomas and seminomas) there was a high level of topoisomerase II α , although expression was still detectable in colon tumours, indicating that expression of this isoform is not sufficient to explain the intrinsic drug resistance of colon tumours. Topoisomerase II β was expressed ubiquitously *in vivo* and was localised in both the nucleoli and the nucleoplasm. This isoform was present in quiescent cell populations, but was expressed at a generally higher level in all tumours and proliferating cells than in normal quiescent tissues. We conclude that topoisomerase II α is a strict proliferation marker in normal and neoplastic cells *in vivo*, but that topoisomerase II β has a much more general cell and tissue distribution than has topoisomerase II α . The apparent upregulation of

topoisomerase II β in neoplastic cells has implications for the response of patients to antitumour therapies that include topoisomerase II-targeting drugs.

The normal tissue distribution in humans of the topoisomerase II α and β proteins using isoform-specific monoclonal antibodies has been studied. In addition, we have analysed representative samples of malignant cells from tumour types in which topoisomerase II inhibitors are routinely used therapeutically. We show that while topoisomerase II α is a strict proliferation marker *in vivo*, topoisomerase II β is expressed widely and in all tissue types, including within quiescent cell compartments. In contrast to some previous reports, we find that topoisomerase II β is detectable both in the nucleoplasm and in nucleoli, and is expressed at a generally higher level in neoplastic than normal tissues.

MATERIALS AND METHODS

Growth and immunocytochemical analysis of cell lines

Cell lines were grown in RPMI-1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 4 mM glutamine, and antibiotics in a humidified atmosphere containing 5% CO₂ at 37°C. For immunocytochemical staining, cytopsin samples were prepared on glass slides using a Shandon cytocentrifuge. The cytopsin samples were then air dried and fixed in PBS containing 3.7% formalin for 15 minutes prior to immunostaining. The cell lines used were as follows:- SUDHL-1 (T-cell lymphoma), MCF-7 (breast carcinoma), SuSa (testicular teratoma), NCI460 (non small cell lung cancer) and CEM (erythro-leukaemia).

Antibodies

The CRB antibody was raised in rabbits to an extreme C-terminal peptide of the human topoisomerase II α protein (ARG-ALA-LYS-LYS-PRO-ILE-
5 LYS-TYR-LEU-GLU-GLU-SER-ASP-GLU-ASP-ASP-LEU-PHE) and was supplied by Cambridge Research Biochemicals (UK). This antibody has been validated in previous studies (Smith and Makinson, 1989; Wells *et al*, 1994). The generation of the 3H10 antibody specific for topoisomerase II β will be described in detail elsewhere (N. Nozaki *et al*,
1 manuscript in preparation). Briefly, a peptide encompassing residues 1583-1601 (SDFPTEPPSLPRTGRARKE) of the deduced human topoisomerase II β sequence (Jenkins *et al*, 1992) was synthesised, conjugated to keyhole limpet haemocyanin, and was injected four times every two weeks into a Balb/c mouse. Antibody secreting cells were
5 screened using a partially purified preparation of topoisomerase II from HL60 cells. The hybridoma 3H10 was cloned and shown to secrete antibody of the IgG2a subtype.

Western blotting

Whole cell extracts from the human lung carcinoma cell line NCI460 and an etoposide-resistant derivative designated NCI460/pV8 were prepared for Western blotting by lysing cells directly in SDS sample buffer (200 μ g protein/ml) prior to separation on a 7% SDS-polyacrylamide gel
5 (Laemmli, 1970). Proteins were then electroblotted at 30 volts for 16 hours onto Hybond-ECL nitrocellulose (Amersham). Detection of topoisomerase II α and β was performed using the CRB antibody (topoisomerase II α -specific) at 1:1000 dilution and the 3H10 antibody (topoisomerase II β -specific) at 1:5 dilution. Enhanced chemi-
luminescence detection was as recommended by the supplier (Amersham),

with the blocking buffer comprising 20 mM Tris-HCl, pH 7.6, 0.9% NaCl, 0.05% Tween-20 and 1% low fat milk powder.

Tissues

A range of normal tissues (tonsil, spleen, lymph node, thymus, skin, pancreas, testis, colon, kidney, liver, brain and lung) and tumours (9 breast carcinomas, 10 colon carcinomas, 13 lung carcinomas, 10 cases of Hodgkin's disease, 13 large cell non-Hodgkin's lymphomas (NHL), 8 cases of lymphocytic lymphoma (CLL) and 8 seminomas of the testis; see Table 2) was obtained from the frozen tissue bank stored at -70°C in the University Department of Cellular Science, John Radcliffe Hospital, Oxford, UK. Cryostat sections of 8 µm were obtained and were mounted on poly-L-lysine coated glass slides. After drying for between 30 minutes and 8 hours, the sections were fixed in PBS containing 3.7% formalin for 15 minutes and then immediately immunostained using an immunoperoxidase Duet kit (Dako, Denmark).

The tumours were classified according to the proportion of labelled cell nuclei as follows: 0-5%, 5-30%, 30-60% and greater than 60%. These were established initially by counting the number of unlabelled and labelled nuclei throughout the section. It was found with experience that this system could be reproduced without formal counting by visual inspection of the section. This was validated by reviewing tumours in the series and comparing visual estimate with the previously established percentages. Tumours were consistently placed within the same proliferation category.

Table 2: Staining of human tumours for topoisomerase II α expression

Tumour type	% of nuclear positive - no of cases				
	n	< 5	5-30	> 30-60	> 60
LYMPHOMAS					
NHL-high grade	4	-	1	1	2
NHL-low grade	7	-	6	1	-
CLL	8	6	1	1	-
Hodgkin's disease	10	2	7	1	-
LUNG					
Squamous	7	-	4	3	-
Adenocarcinoma	4	2	2	-	-
Carcinoid	1	1	-	-	-
Small cell	1	-	-	1	-
SEMINOMA	8	-	-	5	3
COLON	10	-	-	8	2
BREAST	9	8	1	-	-
TOTAL	69	19	22	21	7

Tumours are classified by type and % of nuclei staining positive for topoisomerase II α .

RESULTS

Characterization of topoisomerase II isoform-specific antibodies

Western blotting with the CRB antibody raised to a synthetic peptide from the topoisomerase II α protein sequence (that is not conserved in topoisomerase II β) revealed a single 170 kDa immunoreactive protein consistent with the known size of the topoisomerase II α protein (Figure 9).

This antibody has been shown in previous studies to be specific for the topoisomerase II α isoform (Smith and Makinson, 1989; Wells *et al*, 1994). When the CRB and the 3H10 (which was raised to a non-conserved peptide from the topoisomerase II β sequence) antibodies were mixed and the same filter was exposed simultaneously to both antibodies, a second 180 kDa immunoreactive protein of the size of topoisomerase II β , was revealed (Figure 9). Western blots using the 3H10 antibody alone revealed a single immunoreactive protein of 180 kDa, which co-migrated with topoisomerase II β protein detected with a previously-characterised (Houlbrook *et al*, 1995) rabbit polyclonal anti-topoisomerase II β antiserum raised against recombinant protein (data not shown). Thus, we conclude that the CRB and 3H10 antibodies are specific for the α and β isoforms of topoisomerase II, respectively.

Normal tissue distribution of topoisomerase II α and β in humans

Topoisomerase II α

The anti-topoisomerase II α peptide antiserum, CRB, produced nuclear staining in all of the normal tissues studied with a distribution very similar to that seen with known proliferation-associated antigens, such as Ki-67 (Figure 10). For example, in lymphoid tissue, topoisomerase II- α expressing cells were numerous in the germinal centres, but scarce in mantle zones. In epithelium and testicular tubules, positive staining for topoisomerase II α was present in the basal layers, but not in the more mature superficial cells. In colon and lung, positive staining was present in a minority of basal and alveolar epithelial cells, respectively. In other tissues, including liver, kidney and brain, where the majority of the cells are mature and non-proliferating, positive staining was limited to a few scattered cells. Cytoplasmic staining was noted in some tissues, and was

found in the same cell populations in which nuclear staining was evident.

Topoisomerase II β

- 5 The anti-topoisomerase II β peptide 3H10 antiserum produced positive staining in virtually all cell nuclei within all of the normal tissues studied (Figure 10). A punctate pattern of nuclear staining was evident, which was localised both within nucleoli and dispersed throughout the nucleoplasm. In areas representing proliferating cell populations, such as
10 lymphoid germinal centres, the nucleoli appeared larger, and there was a greater dispersion of immunoreactive material into the surrounding nucleoplasm. In colon there were scattered nuclear dots in most of the cells.

15 *Expression of topoisomerase II α and β in neoplastic tissues*

Topoisomerase II α

- The staining pattern for topoisomerase II α protein seen in the range of
20 tumours examined reflected that of the normal tissues described above. The CRB antibody gave a pattern of nuclear staining that strongly correlated with that seen with antibodies to the established proliferation marker, Ki-67 antigen. Of particular note was the striking positivity of the abnormal mono- and multi-nucleate cells in cases of Hodgkin's disease
25 (Figure 10). Cytoplasmic staining with the CRB antibody was noted and was generally more evident in the tumour biopsies than it was in the normal tissue samples.

- The proportion of tumour cells staining positive for topoisomerase II α
9 ranged from less than 5% to more than 60%, and this was related to

tumour type and grade (Figure 10 and Table 2). For example, high grade lymphomas had a higher proportion of positively-staining cells than did low grade lymphomas and lymph nodes from patients with chronic lymphatic leukaemia. For non-small cell lung cancers, the squamous tumours had a higher proportion of positively staining cells than did adenocarcinomas or carcinoid tumours. Seminomas showed the highest percentage of cells staining positive for topoisomerase II α , while expression was generally low in breast cancers. The intrinsically drug resistant colon tumours analysed showed a generally high percentage of cells staining positive for topoisomerase II α (Table 2).

Topoisomerase II β

The topoisomerase II β -specific antibody 3H10 produced granular nuclear staining in virtually all of the cell types in every tumour analysed. No direct association with proliferative index (and therefore with topoisomerase II α expression) was evident, although there was a generally higher intensity of staining in tumour tissue than that seen in normal tissues (Figure 10). In each tumour sample, a minimum of 50% of the cells stained positive for topoisomerase II β , although in most cases more than 90% of cells expressed topoisomerase II β . As with normal tissues, staining within both nucleoli and in the nucleoplasm was evident with the 3H10 antibody, but more intense staining coincided with nucleolar structures. In lymphoid neoplasms, only a limited amount of cytoplasmic staining was evident, but in the seminomas and epithelial neoplasms (lung colon and breast cancer), many cells had a low level of detectable cytoplasmic staining.

*Expression of topoisomerase II α and β in cell lines***Topoisomerase II α**

- 5 Staining of cell lines with the CRB antibody showed nuclear staining with nucleolar accentuation. The mitotic figures were strongly positive (Fig 11). There was some cytoplasmic staining, but this was weak.

Topoisomerase II β

- 0 Staining with 3H10 on all of the lines showed a different pattern from that of CRB. The pattern was nuclear, but showed a granular distribution (Fig 11). In one cell line (SUDHL-1), the cells undergoing mitosis (or cells that had just divided) showed staining in the cytoplasm as granules with
5 a negatively-staining nucleus.

DISCUSSION

- We have analysed the expression and distribution of the α and β isoforms
0 of topoisomerase II in normal and neoplastic human tissues. Topoisomerase II α was detected in the proliferative compartment of all normal tissues. In contrast, topoisomerase II β was detectable in virtually all cells, irrespective of their proliferative status, although some evidence for modest upregulation in proliferating cells was obtained.

.5

REFERENCES FOR EXAMPLE 2

- Houlbrook *et al* (1995) *Brit. J. Cancer* **72**, 1454-1461.
- Jenkins *et al* (1992) *Nucl. Acids Res.* 5587-5592.
- 5 Laemmli, U.K. (1970) *Nature* **227**, 680-685.
- Smith, P.J. and Makinson, T.A. (1989) *Cancer Res.* **49**, 1118-1124.
- Wells *et al* (1994) *J. Biol. Chem.* **269**, 29746-29751.

Example 3: Genetic constructs expressing cytosine deaminase or thymidine phosphorylase from the topoisomerase II α promoter

The -144 to +93 promoter element from the human topoisomerase II α gene is linked to the *E. coli* cytosine deaminase gene (or to thymidine phosphorylase gene/cDNA) in several different vector systems. A simple plasmid vector is used and transfected into cell lines using standard techniques. The expression of the pro-drug activating gene is assessed by quantifying ability to kill the transfected cells using 5-fluorocytosine (which is converted to 5-fluorouracil by cytosine deaminase) or 5'-deoxy-5-fluorouridine (which is converted to 5-fluorouracil by thymidine phosphorylase). The cell lines are Saos-2 (p53null) or Saos-2 expressing p53^{wt} or temperature-sensitive p53. Clonogenic survival curves are determined at 37°C for the first two cell lines and at both 37°C and 32°C for the latter host cell line. Because transfection is a relatively inefficient process, the transfectants are extracted from the bulk of the untransfected population by use of the pHook system. In this, the cells are co-transfected with cDNA encoding a cell surface marker which permits transfectants to be recovered using magnetic beads coated in antibody to the surface marker. In all cases cell survival in cases where p53 is wild-type and where it is mutant are compared. Survival rates will also be compared in cell populations that are in exponential phase and in those that are quiescent due to plating at high density and induction of confluence-induced growth arrest. Retroviral vectors containing the genetic constructs are made and these vectors give near 100% infection frequencies, but only replicate in proliferating cells and as such are mainly useful for confirming the p53-mediated regulation of the promoter. Nevertheless, following infection, cells can be grown to confluence to analyse proliferation-specific regulation of gene expression.

Example 4: Transient co-transfection assays using the topoisomerase II α promoter and luciferase reporter gene

The topoisomerase II α promoter element (-144 to +93) is linked to a luciferase reporter gene. This genetic construct is co-transfected with different dosages of a genetic construct expressing wild-type or mutant (143, 248 or 216) p53 genes into a p53-minus human leukaemia cell line, K562.

In a parallel experiment, genetic constructs in which the SV40 early promoter or the HSV-tk promoter or the CMV promoter or the human hsp70 promoter are linked to the luciferase reporter gene are used thus allowing direct comparison between the topoisomerase II α promoter and other p53-regulatable promoters.

Example 5: Inclusion of the topoisomerase II α promoter element in a "dual control unit"

A "dual control unit" analogous to those described in WO 97/12970 is made. Genetic unit I contains a topoisomerase II α promoter (-144 to +93) with *lac* operator driving a renilla luciferase (rl) reporter. Genetic unit II contains a tkGC3p53 promoter (contains a consensus p53 binding site upstream of the minimal HSV-thymidine kinase promoter) driving a firefly luciferase reporter. Insulator sequences from chick β -globin gene are used.

The genetic construct is co-transfected into K562 cells in the presence or absence of wild-type p53 and the activities of both luciferases are measured.

Details of the luciferase reporter genes, tkGC3p53 promoter and chick β -globin insulator sequences are described in WO 97/12970.

CLAIMS

1. A genetic construct comprising a promoter element responsive to a tumour suppressor gene product and a cytotoxic gene wherein the promoter element is substantially inactive in a cell which contains said tumour suppressor gene product encoded by a wild type tumour suppressor gene and is active in a cell which contains a variant said tumour suppressor gene product encoded by a mutant tumour suppressor gene, or does not contain said tumour suppressor gene product.
2. A genetic construct according to Claim 1 wherein the promoter element is active in a proliferating cell but is substantially inactive in a non-proliferating cell.
3. A genetic construct according to Claim 1 or 2 wherein the tumour suppressor gene is p53.
4. A genetic construct according to any one of Claims 1 to 3 wherein the promoter element comprises the human topoisomerase II α promoter or a portion thereof.
5. A genetic construct according to Claim 1 wherein the tumour suppressor gene is Rb.
6. A genetic construct according to any one of the preceding claims wherein the cytotoxic gene is cytosine deaminase or thymidine kinase or thymidine phosphorylase.
7. A genetic construct according to any one of the preceding claims

further comprising a genetic element to enhance the expression of the cytotoxic gene product.

8. A genetic construct according to any one of the preceding claims further comprising a tissue- or cell-type- or further tumour selective genetic element.
9. A genetic construct according to any one of the preceding claims comprising DNA.
10. A genetic construct according to any one of Claims 1 to 8 comprising RNA.
11. A genetic construct according to any one of the preceding claims adapted for delivery to a cell.
12. A virus or virus-like particle comprising a genetic construct according to any one of Claims 1 to 10.
13. A composition comprising a genetic construct according to any one of Claims 1 to 10 and means for introducing said genetic construct into a cell.
14. A genetic construct according to Claim 1 to 11 for use in medicine.
15. A virus or virus-like particle according to Claim 12 for use in medicine.
16. A composition according to Claim 13 for use in medicine.

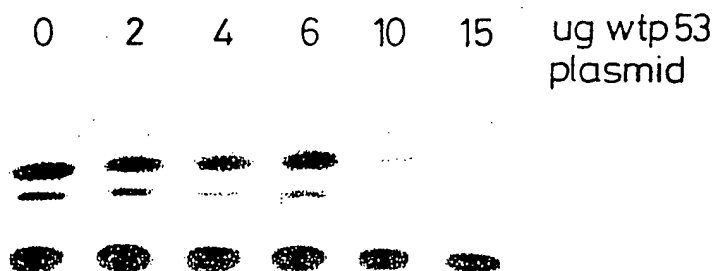
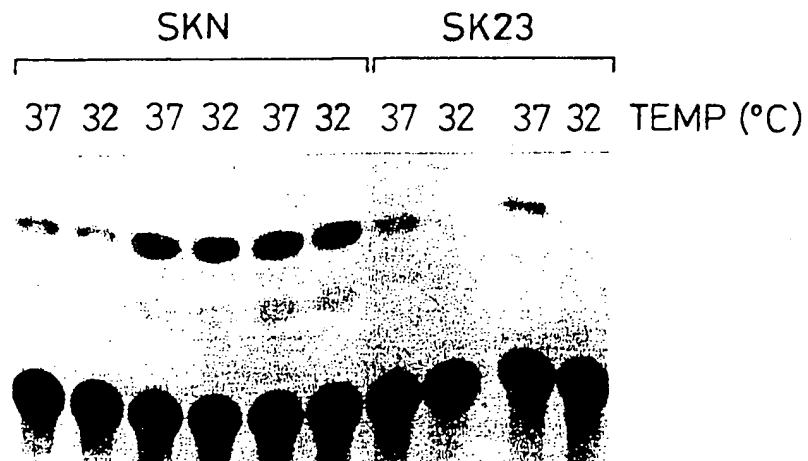
17. A pharmaceutical composition comprising a genetic construct as defined in any one of Claims 1 to 11; or a virus or virus-like particle as defined in Claim 12; or a composition as defined in Claim 13 and a pharmaceutically acceptable carrier.
18. A method of treating a host with a cancer, or a host which host may develop cancer, the method comprising administering to said host a therapeutically effective amount of a genetic construct according to any one of Claims 1 to 11 or a virus or virus-like particle according to Claim 12 or a composition according to Claim 13.
19. A method of treatment according to Claim 18 wherein said cytotoxic gene is indirectly cytotoxic further comprising administering to said host an additional component, acted upon by said indirectly cytotoxic gene or its product, and which becomes cytotoxic by said action.
20. A method of treatment according to Claim 18 or 19 wherein prior or during administration of said genetic construct or virus or virus-like particle or composition the tumour suppressor gene status of said cancer is determined.
21. Use of a genetic construct according to any one of Claims 1 to 11 or a virus or virus-like particle according to Claim 12 or a composition according to Claim 13 in the manufacture of a medicament for treating cancer.
22. A therapeutic system comprising a genetic construct according to any one of Claims 1 to 11, or a virus or virus-like particle

according to Claim 12 or a composition according to Claim 13 wherein said cytotoxic gene is indirectly cytotoxic and an additional component acted upon by said indirectly cytotoxic gene or its product and which becomes cytotoxic by said action.

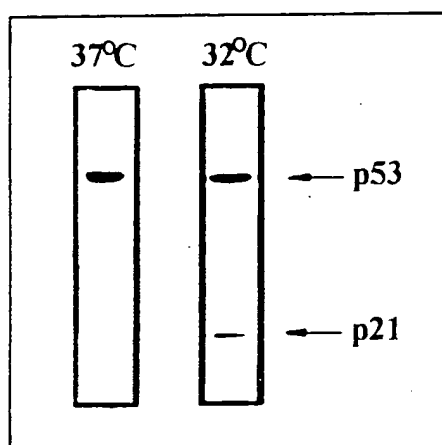
5

23. A therapeutic system comprising a genetic construct according to any one of Claims 1 to 11 or a virus or virus-like particle according to Claim 12 or a composition according to Claim 13 and means for determining the tumour suppressor gene status of a
- 10 tumour in a patient.

1/11

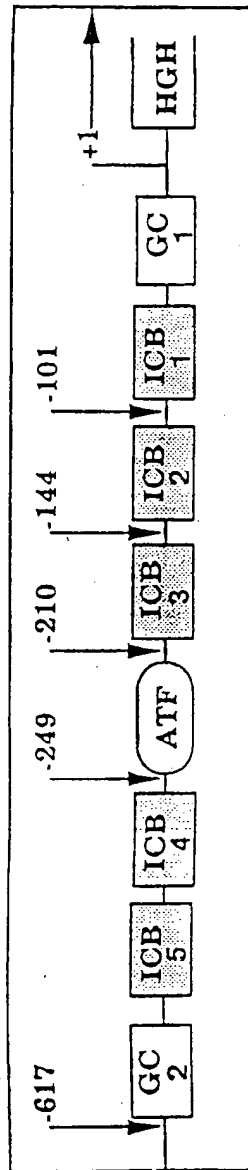
*Fig. 1**Fig. 2*

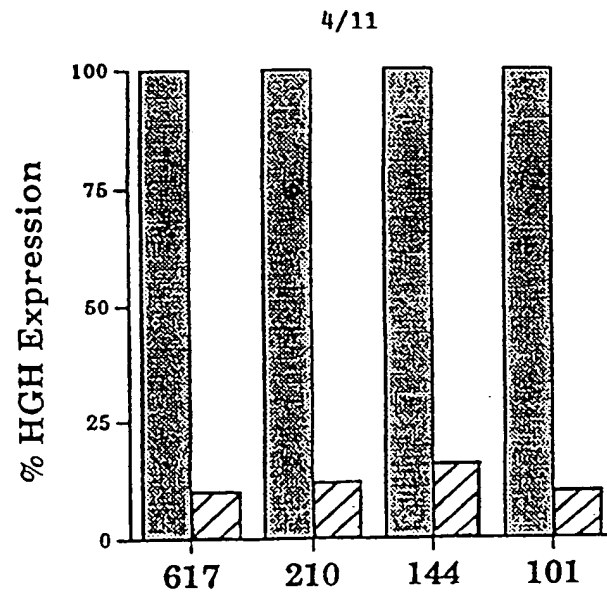
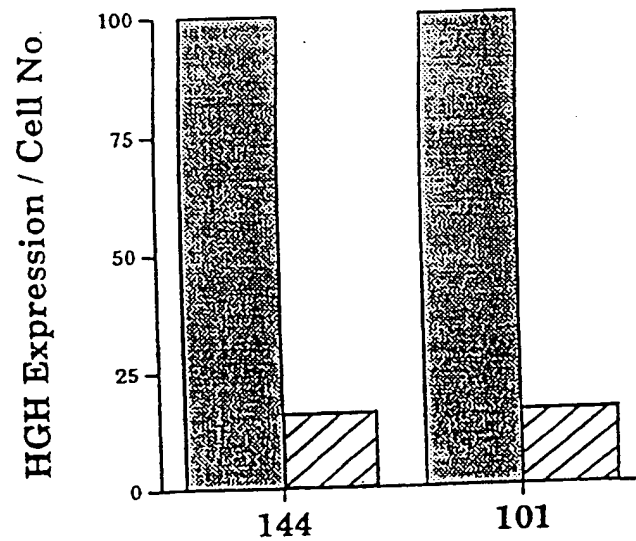
2/11

*Fig. 3*

SUBSTITUTE SHEET (RULE 26)

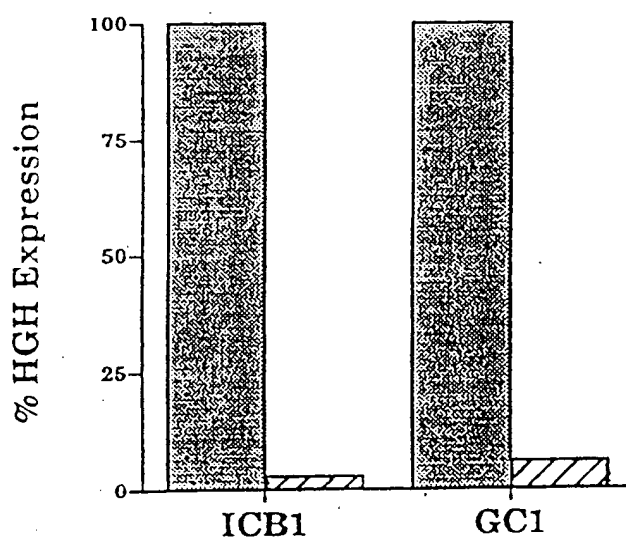
3/11

*Fig. 4a*

*Fig. 4b**Fig. 4c*

SUBSTITUTE SHEET (RULE 26)

5/11

*Fig. 5*

6/11

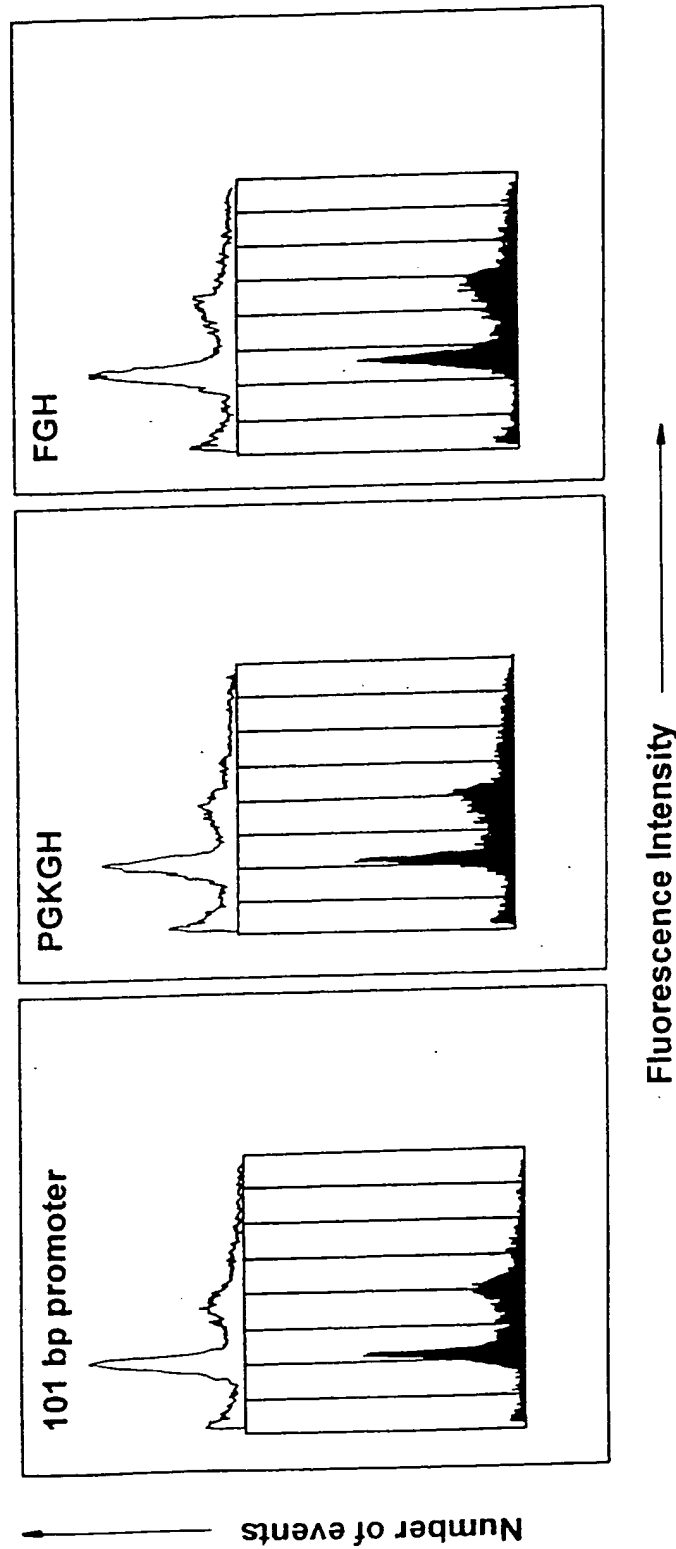
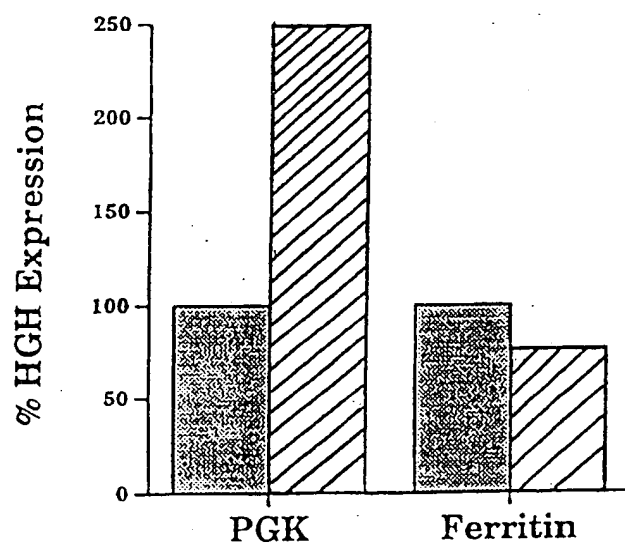


Fig. 6

SUBSTITUTE SHEET (RULE 26)

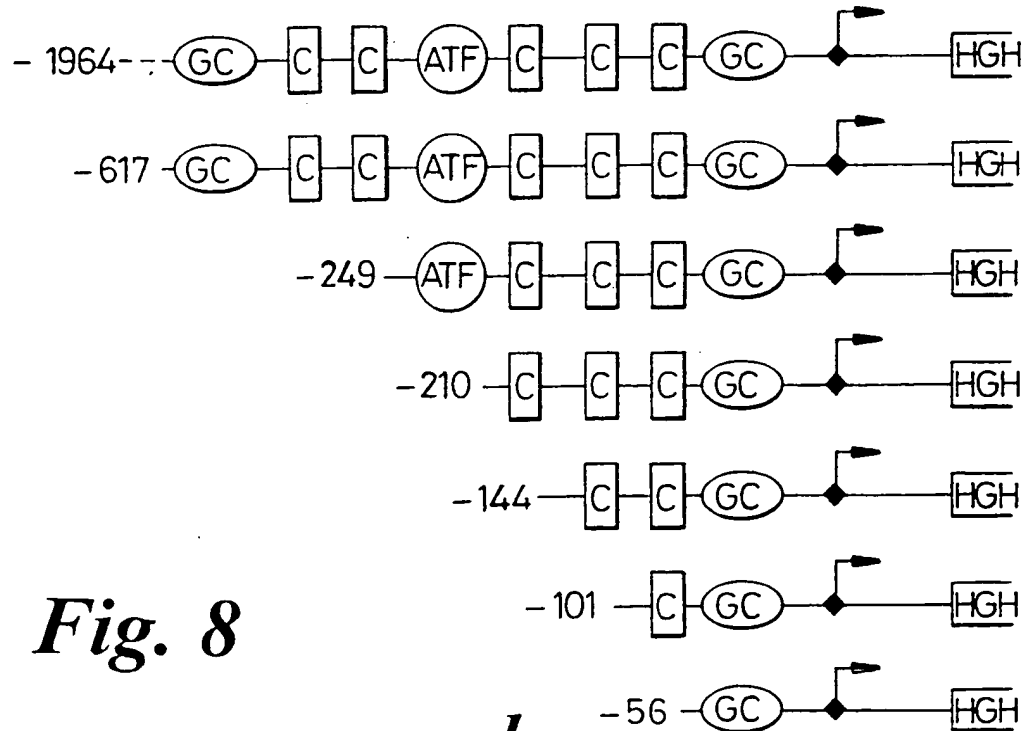
7/11

*Fig. 7*

8/11

GC2

-617 AGCCACCGCACACAGCCTACTTTTATTTCTTTGAAAAATGAATTCGAGGGTAAAGGGGCG
 -557 GGGTTGAGGCAGATGCCAGAATCTGTTTCGCTTCAACCAAGCAGCCAGGCTGCCTGTCCAG
 -497 AAAGCCGGCACTCAGTTTCTCAGGAAAACGAAGCTAAGGCTCCCATTCCTCGCTAAC
 -437 AACGTCAGAACAGAGGACAGTTTTTAGATTTTCAGGGATCTTAAATAGATTGGCAGTTCCT
 -377 GGAGAATAAACATCCTTTGCTTTTCTCCTGCACACTTTTGCCTCAGGCCACCCCTTCCCG
 -317 CTTCCAAAGCCCATCTCTTCCAAGCTTTCGCACGAGAAAACAAGTGAGCCCTTCTCATT
 -257 GGCAGATTCCCTGTCAATCTCTCCGCTATGACGCCGAGTGGTGCCTTTTGAAGCCTCTC
 -197 TAGTCCCGCCTCCCTAACCTGATTGGTTTATTCAAACAAACCCCGGCCAACTCAGCCGTT
 -137 CATAGGTGGATATAAAAGGCAAGCTACGATTGGTTCTTCTGGACGGAGACGGTGAGAGCG
 -77 AGTCAGGGATTGGCTGGTCTGCTTCGGGCGGGCTAAAGGAAGGTTCAAGTGGAGCTCTCC
 -17 TAACCGACGCGCTCTGTGGAGAAGCGGCTTGGTCGGGGTGGTCTCGTGGGGTCTCGCC
 +44 TGTTTAGTCGCTTTCAGGGGTTCTTGAGCCCCTTCACGACCGTCACCATG

a*Fig. 8**b*

SUBSTITUTE SHEET (RULE 26)

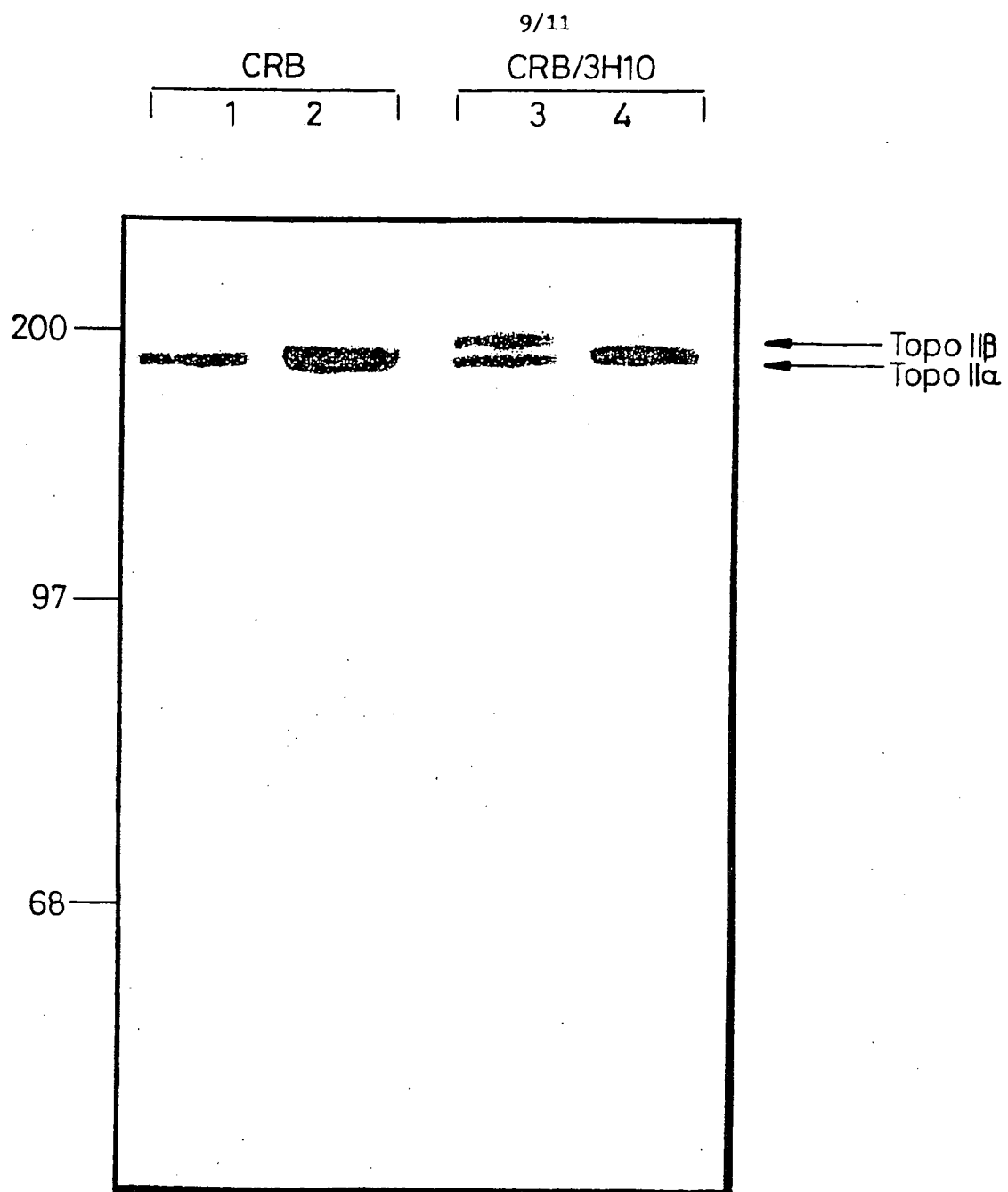
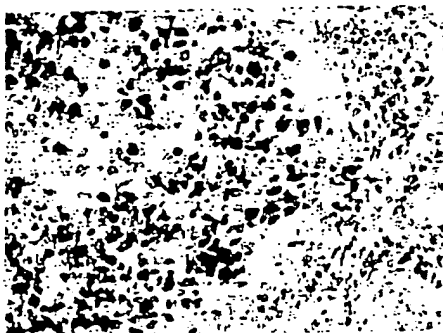
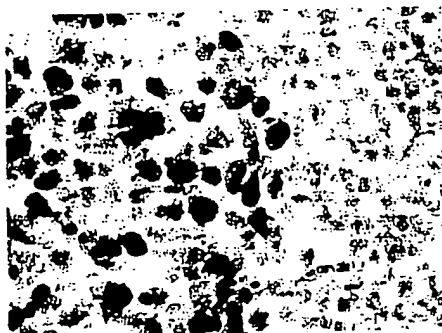
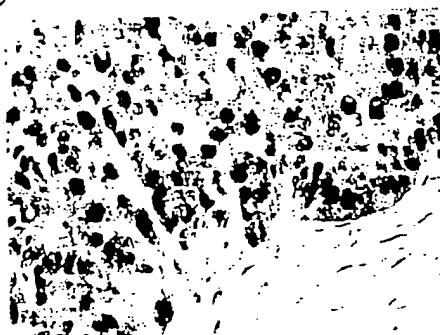
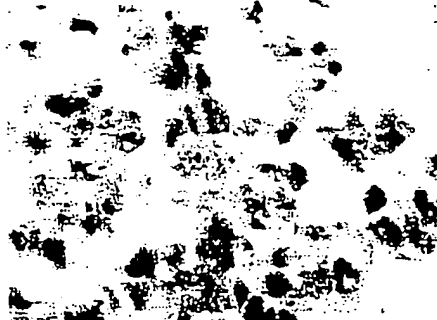
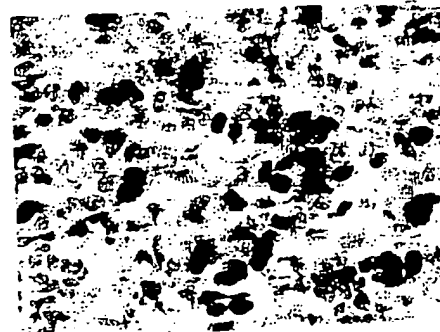
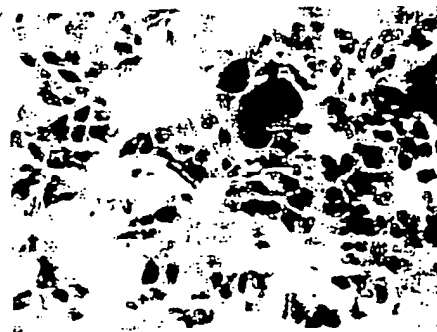


Fig. 9

10/11

a*b**c**d**e**f**g**h**Fig. 10*

SUBSTITUTE SHEET (RULE 26)

11/11

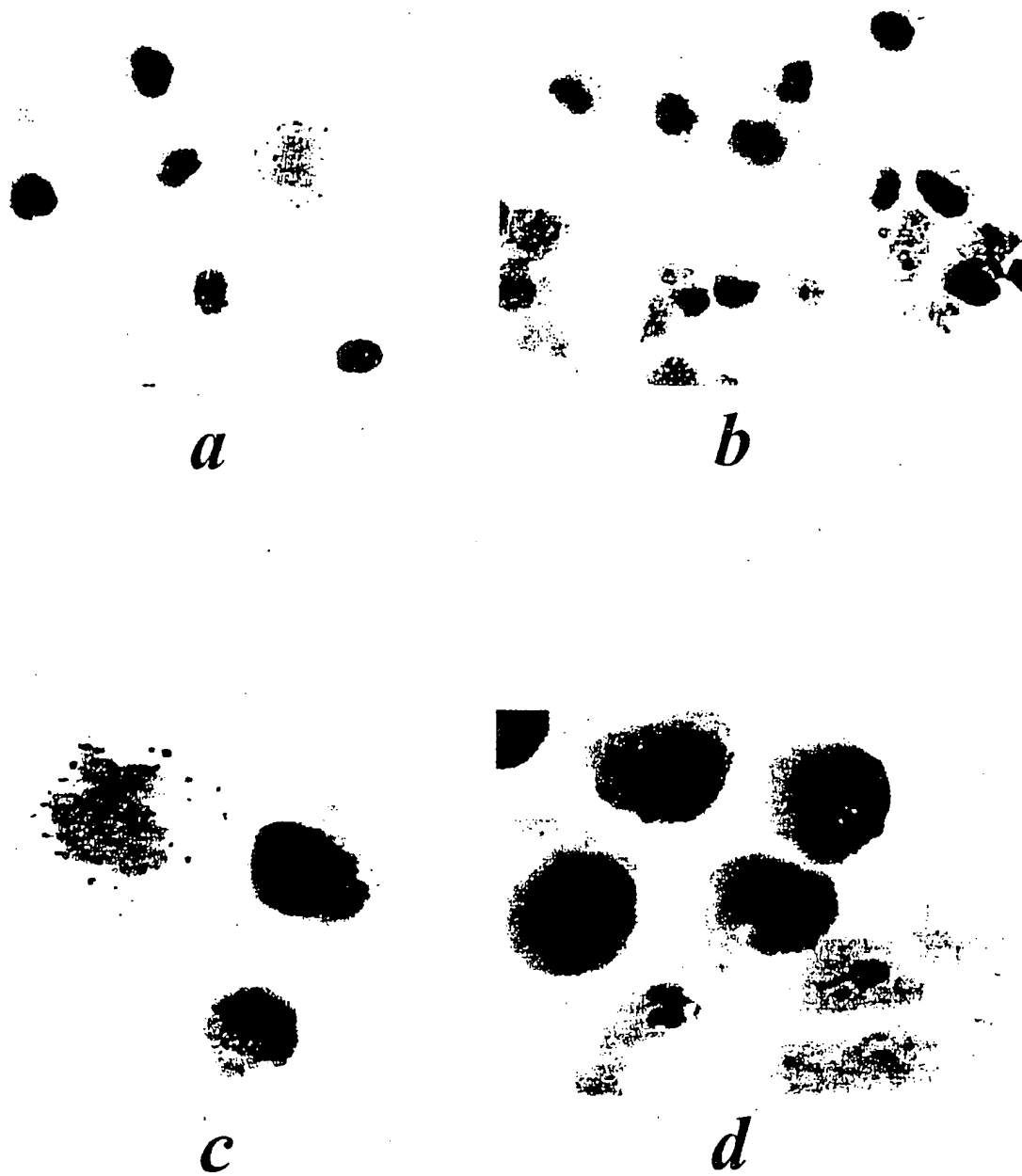


Fig. 11

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 98/00551

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/63 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GB 2 305 920 A (CANCER RES CAMPAIGN TECH) 23 April 1997 cited in the application	1,2,6-9, 11-23
Y	see page 46, line 10 - page 61 ---	3
Y	TSUTSUMI-ISHII Y ET AL: "RESPONSE OF HEAT SHOCK ELEMENT WITHIN THE HUMAN HSP70 PROMOTER TO MUTATED P53 GENES" CELL GROWTH AND DIFFERENTIATION, vol. 6, no. 1, January 1995, pages 1-8, XP000616356 see page 5, right-hand column, paragraph 2 --- -/--	3

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

22 June 1998

Date of mailing of the international search report

06.07.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

Internatio	Application No
PCT/GB	98/00551

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HUBER B E ET AL: "VDEPT: AN ENZYME/PRODRUG GENE THERAPY APPROACH FOR THE TREATMENT OF METASTATIC COLORECTAL CANCER" ADVANCED DRUG DELIVERY REVIEWS, vol. 17, no. 3, 5 December 1995, pages 279-292, XP000654839 see figure 1</p> <p style="text-align: center;">---</p>	1,2,6-23
A	<p>HARRIS J D ET AL: "GENE THERAPY FOR CANCER USING TUMOUR-SPECIFIC PRODRUG ACTIVATION" GENE THERAPY, vol. 1, no. 3, May 1994, pages 170-175, XP000654731 see abstract</p> <p style="text-align: center;">---</p>	1,2,6-23
A	<p>SANDRI MI ET AL: "P53 REGULATES THE MINIMAL PROMOTER OF THE HUMAN TOPOISOMERASE II ALPHA GENE" NUCLEIC ACIDS RESEARCH, vol. 24, no. 22, 15 November 1996, OXFORD GB, pages 4464-4470, XP002068824 cited in the application see page 4469, right-hand column, paragraph 1</p> <p style="text-align: center;">-----</p>	1-4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00551

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 18-20 are directed to a method of treatment of the human or animal body, the search has been carried out and based on the alleged effects of the genetic construct.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00551

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2305920 A	23-04-1997	AU 7138696 A	28-04-1997
		WO 9712970 A	10-04-1997
